CULTURE AND GROWTH CHARACTERIZATION OF HUMAN MESENCHYMAL STEM CELLS FROM DENTAL PULP

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

APRIL 2011

CERTIFICATE

This is to certify that this dissertation titled **"CULTURE AND GROWTH CHARACTERIZATION OF HUMAN MESENCHYMAL STEM CELLS FROM DENTAL PULP"** is a bonafide record of work done by **Revathi S** under our guidance during her postgraduate study period between 2008-2011.

This dissertation is submitted to **THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI**. It has not been submitted (partial or full) for the award of any other degree or diploma.

Dr. K. Ranganathan, MDS, MS. (Ohio), PhD
Professor & HOD
Department of Oral and Maxillofacial Pathology
Ragas Dental College & Hospital
Chennai.

Dr. M. Uma Devi, MDS
Professor
Department of Oral and Maxillofacial Pathology
Ragas Dental College & Hospital
Chennai.

Dr. S. Ramachandran, MDS
Principal
Ragas Dental College & Hospital
Chennai

CONTENTS

Acknowledgement

'God is truth and light His shadow'

I thank the Lord for making it all possible, for being my guiding light.

With the deepest gratitude I wish to thank every person who came into my life and inspired, touched and illuminated me through their presence.

Words can do little justice to express my deep felt gratitude to my postgraduate teacher and mentor, **Dr. K Ranganathan, MDS, MS (Ohio), PhD, Professor and Head of the Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital**, for guiding me at every stage, for his encouragement, for selflessly sharing his knowledge, for inculcating in me a keen interest in cell culture and above all, for having faith in me. His presence has been instrumental in making me who I am today.

I extend my gratitude to **Dr. Uma Devi K Rao, MDS, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital**, for her support and guidance towards the completion of my thesis, for generously sharing her wisdom and making the study of pathology interesting and an absolute pleasure.

I whole-heartedly thank **Dr. Elizabeth Joshua, MDS, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital**, for helping me throughout my post graduate curriculum. Her cheerful nature and optimism provided me with the impetus to complete my thesis.

I thank **Dr. T. Rooban, MDS, Associate Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital** for his patience and valuable advice that helped in shaping my study and postgraduate curriculum.

My heartfelt gratitude to **Dr. Deepu George Mathew, MDS, Senior Lecturer, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital** who encouraged me to take up this thesis. I also thank him for tirelessly guiding me throughout the period of my dissertation. His patience and constant support were invaluable.

I extend my thanks to **Dr. K. M Vidya, Dr. P Jayanthi and Dr Lavanya. N, Senior Lecturers, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital** for their encouragement and advice throughout my postgraduate curriculum.

I thank **Dr Veerabahu**, **MDS, Professor and Head of the Department of Oral and Maxillofacial Pathology, Dr M Jayanthi MDS, Professor and Head of the Department of Pedodontics,** and **Dr Ashwin Mathew George, MDS, Professor, Department of Orthodontics, Ragas Dental College & Hospital** for being extremely supportive and helping me with the sample collection.

I thank **Mrs Deepa S**, statistician, for helping me with the statistical analysis.

I extend my gratitude to **Mrs Kavitha Wilson**, **M.Sc,** for her support, advice and encouragement throughout my study.

I thank **Mr Rajan** for his help and encouragement throughout my postgraduate curriculum.

I am extremely grateful to my senior, **Dr Skariah** and batch mates, **Dr Aesha, Dr Divya, Dr Jeyapreetha, Dr Shahela** and **Dr Sreeja** for their constant support, without which I would not have been able to complete my study.

Stem cells are defined as ‘cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation.’ [1]

Stem cell research has been gaining momentum for the past two decades and in the past few years it has generated significant interest due to the success achieved in culturing human embryonic stem cells, and in manipulating their differentiation *in vitro*.[2] Methods developed to assess and identify stem cells within a heterogeneous population using microarrays and comparison of data sets *in silico* have further facilitated their clinical application.[3]

The human body contains several loci or compartments called ‘stem cell niches’, inhabited by a significant number of stem cells. The dental pulp, contained within the ‘sealed niche’ of the pulp chamber, is an extremely rich site for stem cells. Stem cells in dental pulp reside in a perivascular micro-environment, where they are quiescent but have the potential to express basic stem cell characteristics.[4, 5, 6, 7, 8] These stem cells are called DPSC (dental pulp stem cells), in permanent teeth and SHED (stem cells from human exfoliated deciduous), in deciduous teeth.

DPSC and SHED are mesenchymal stem cells similar to the first mesenchymal stem cells isolated from bone marrow, (bone marrow mesenchymal stem cells; BM-MSCs) [4,9,10,11,12,13,14,15] that have the capacity to form single-cell-derived colony clusters called Colony Forming Unit-fibroblast (CFU-F) *in vitro*.[16,17] Accumulated knowledge of the phenotypic characteristics of BM-MSCs has contributed significantly to the isolation of putative stem cell populations from dental pulp of human permanent teeth and deciduous teeth.

As stem cells are self-renewing and multi-potent, they can potentially generate any tissue in their lifetime. The ability to expand stem cells and induce their differentiation in culture is a key property for their use in therapy, and is of great clinical interest in many diseases including Parkinson's[19], cardiovascular disease[18] and correction of neural degeneration following brain injury.[19]

In dentistry, stem cells are being used for the regeneration of dentin, creation of biologically viable scaffolds for the replacement of orofacial bone and cartilage, [20] craniofacial regeneration-including the temporomandibular joint, [21] and regeneration of periodontal ligament and cementum.[22] As dental pulp derived stem cells produce neurotropic factors, they have the potential to be used in neural regeneration.[19]

Clinically, DPSC have an advantage over other types of adult stem cells in that they are easy to access and are extracted during life. Also, exfoliated deciduous teeth can be secured at a young age and the cells obtained, stored for future use. A stem cell bank can be created from the DPSC / SHED without using procedures that are invasive.

The first step in the use of stem cells for therapy is their characterization. This is achieved by studying the phenotypic features, growth pattern and markers of cellular differentiation.

The present study was done to isolate and expand the mesenchymal cell population from the dental pulp of both deciduous and permanent teeth, to ascertain the feasibility, standardize the procedure and characterize their growth properties and morphology.

Aim of the study-

To isolate, culture and study the morphology and growth characteristics of mesenchymal stem cells from the dental pulp of permanent teeth (DPSC) and exfoliated deciduous teeth (SHED).

Objectives of the study-

1. To isolate and culture mesenchymal stem cells from permanent teeth (DPSC) and exfoliated deciduous teeth (SHED) using enzyme disaggregation technique.

2. To compare the phenotypic characteristics of cells obtained from the dental pulp of permanent teeth (DPSC) and exfoliated deciduous teeth (SHED).

3. To ascertain the population doubling time of cells obtained from the dental pulp of permanent teeth (DPSC) and exfoliated deciduous teeth (SHED).

4. To ascertain the capacity to form Colony Forming Units (CFUs) of cells obtained from the dental pulp of permanent teeth (DPSC)

Hypothesis-

The pulp tissues of permanent and deciduous teeth are viable sources of phenotypically similar mesenchymal stem cells.

Materials for tissue Culture:

Reagents

Growth medium-

1. Mesenchymal Stem Cell Medium (MSC Medium)-

   α- modified minimal essential medium (*α*-MEM)

   Fetal Bovine Serum (Invitrogen ^{TM})

   Antibiotics-

   - Penicillin-100 IU/ml.

   -Streptomycin-100µg/ml.

2. D-PBS (Potassium chloride-0.2g/l, Potassium phosphate monobasic-0.2g/l,Sodium chloride-8g/l, Sodium phosphate dibasic-1.15g/l)
3. Distilled water.
4. De-ionized water.
5. Collagenase (type I, filtered) (CLS-1- Worthington Biochemical Corporation ^{TM})
6. Dispase (neutral protease, grade II) (Roche ^{TM})
7. Trypsin 1:125. (Tissue culture grade, Hi media ^{TM})
8. Ethylene-di-amine-tetra-acetic acid. (Hi Media ^{TM})

Equipment

1. Culture dishes. (Tarsons™)
2. 24-well plates. (Cell star ™)
3. Disposable pipettes and pipette tips.
4. Glass pipettes.
5. BP blade no. 15
6. Centrifuge tubes.
7. Leak-proof screw-cap vials.
8. Scott Duran bottles.
9. Laminar flow cabinets.
10. Carbon dioxide incubator. (Thermo electron Corporation. Forma series II water jacketed-HEPA class 100)
11. Phase contrast microscope. (Olympus CKX41 ™)
12. Digital camera. (Kodak AF3X, 8.2 mega pixels, 3x optical zoom)
13. Improved Neubauer counting chamber.
14. Laboratory centrifuge. (R-86 Remi ™)
15. Cyclomixer. (C101 Remi ™)
16. Electronic balance. (Dhona 200D ™)
17. Prabivac vaccum pump.
18. Cellulose acetate filter (pore size 0.2µm)
19. Autoclave
20. Hot air oven
21. Micromotor (Marathon ™)
22. Contra-angled Hand piece (NSK ™)
23. Carborundum discs

24. Chisel
25. Mallet

Methodology-

Tissue collection

Permanent teeth-

Impacted third molars/premolars extracted for orthodontic reasons from patients attending Ragas Dental College and Hospital, Chennai.

Deciduous teeth-

Non-infected, exfoliating deciduous teeth extracted in the Department of Pedodontia, Ragas Dental College and Hospital, Chennai.

Consent was obtained from patients above the age of eighteen years and from the parents of children for the collection of teeth [Annexure I].The study was approved by the Institutional Review Board, Ragas Dental College and Hospital, Chennai.

Transportation of tissue to laboratory for culturing

Teeth extracted under sterile condition were transferred to serum-free α-Minimal Essential Medium(α-MEM), with added antibiotics (Penicillin-100 IU, Streptomycin-100µg/ml), at a pH of 7.2 to 7.4 and maintained at 4°C with the help of ice-packs. They were transported in leak-proof, sterilized culture vials.

Protocol for isolation of dental pulp: [23]

a. Tooth surface was cleaned well by washing thrice with Dulbecco's Phosphate Buffered Saline (D-PBS).
b. Grooves were placed around the cemento-enamel junction with a carborundum disc and ice-cold D-PBS irrigation to avoid heating while cutting.
c. Tooth was split with chisel and mallet to expose the pulp chamber.
d. The pulp tissue was obtained from the pulp chamber with the help of forceps and spoon excavator and put into 2ml of Mesenchymal Stem Cell (MSC) medium on a Petri dish (60mm diameter) to avoid it becoming dry.

Protocol for primary culture of dental pulp cells: [23]

a. The dental pulp tissue was minced into tiny pieces with a surgical blade.
b. The tissue was immersed into a mixed collagenase (2mg) and dispase (1mg) solution in Dulbecco's Phosphate Buffered Saline
c. It was incubated at 37^0C for up to 60 minutes and mixed well intermittently.
d. Cells were centrifuged at 2400rpm for 5 minutes.
e. The supernatant was removed and the pellet re-suspended with MSC medium.
f. The cells were cultured in MSC medium at 37^0C and 5 % CO_2 in the incubator.

Forty-eight hours after the cell isolation, the culture media was discarded and fresh media added to the Petri dish. Media change was repeated every third day until cell confluence was reached.

Subculture

Five to seven days after the cell isolation, colonies were identified in the culture plates, where the cells had a typical spindle / fibroblastoid shape. The cells were sub-cultured after they reached 90% confluency.

Protocol for Subculture: [23]

a. The culture was examined carefully for signs of deterioration or contamination.
b. The media was discarded from the plate.
c. Two washes with 2ml D-PBS was done to remove any residual serum.
d. 1ml trypsin 0.25% with EDTA 0.05% was added to the Petri dish (60mm diameter).
e. The monolayer was checked under the microscope to see whether the cells were rounding
f. The plate was tapped at the bottom until all the cells were detached.
g. Cells suspended in trypsin was collected in a centrifuge tube and centrifuged at 2400 rpm for 3 minutes.

h. Supernatant obtained after centrifugation was discarded. To the remaining cell pellet, the medium was added and cells were dispersed by repeated pipetting.
i. The cells were counted in a haemocytometer.
j. The cell suspension was diluted to appropriate seeding concentration by adding adequate volume of medium in a culture plate.

The plates were closed and returned to the incubator.

Protocol for growing and fixing of cells on APES (3-aminopropyl-triethoxy-silane) **coated slides**

Slides soaked in soap-water for 2 hours

⇓

Slides washed thrice in tap water

⇓

Soaked overnight in 1/10 N hydrochloric acid

⇓

Slides washed thrice in distilled water

⇓

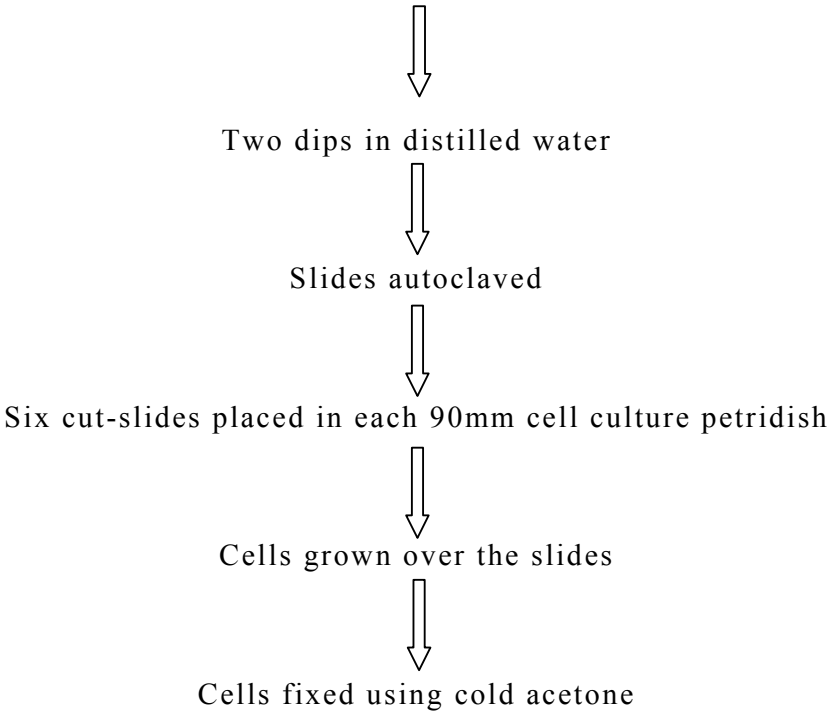
Slides baked in hot-air oven for 4 hours at 60ºC

⇓

Slides dipped in 50ml acetone for 2 minutes

⇓

In 2% APES for 5 minutes



Cell culture studies:

A. Phenotypic characterization was done as follows:

i. F1, F2 and F3 phenotypes [24, 25]

1. Cell lines from the fourth passage were plated on three 60 mm tissue culture Petri dishes at a concentration of 0.5 x 10^4 cells /ml.
2. Using a phase contrast microscope (20x magnification), the cells were observed and counted for eight consecutive days and classified morphologically as F1, F2 and F3 fibroblastoid cells based on the description given by Mollenhauer and Bayreuther in 1986 on rat skin fibroblasts.[24]
3. Thirty cells were randomly studied in each tissue-culture Petri dish giving a total of ninety cells per cell line.

ii. Fibroblastoid and Epithelioid

1. Six days after primary culture, ten cells were randomly counted from both permanent and deciduous tooth pulp cultures.
2. The cells were classified as fibroblastoid, if they had a spindle-shaped morphology and "epithelioid " [24,25] if they appeared round.
3. The number of fibroblastoid and epithelioid cells was estimated over a period of fifteen consecutive days.

B. Estimation of growth curve and its derivatives [26]

1. Cells were inoculated at 1.2 x 10^4 cells /ml/well on 24-well plates
2. After overnight attachment, cells from 3 randomly selected wells were trypsinized and counted using a haemocytometer.
3. The medium was changed on 3rd and 6th days.
4. The count was repeated every 24 hours for 8 days.
5. Cells from each well were counted thrice to avoid error.
6. The averages of daily cell counts of each well were used to plot the growth curve.
7. The seeding cell count and cell count on the first day i.e. 12 hours of seeding used to estimate the seeding efficiency in percentage by using the equation

$$\frac{\text{Cell count/well/ml after 12 hours X 100}}{\text{Seeding cell count/well/ml}}$$

8. The growth curve was plotted and population doubling time (PDT) derived from the exponential growth phase.

C. Clonal assay and estimation of colony forming efficiency [27]

1. Fifth passage cells were seeded at 40 cells/40mm plate in triplicate.
2. At the end of two weeks, cells were fixed in methanol and stained with Crystal violet.
3. Colonies greater than 2mm in diameter were counted.
4. Percentage colony forming efficiency =

$$\frac{\text{Total no. of colonies X 100}}{\text{Initial no. of cells seeded}}$$

Statistical Analysis

Data analysis was done using SPSS ^{TM} (Statistical Package for Social Science) version 10.0.5.

Linear regression analysis was performed

- To derive the slope from growth curves: permanent tooth pulp derived cell lines (14^{th} and 15^{th}) and deciduous tooth pulp derived cell line(10^{th})

Correlation coefficients were determined to analyze

- F1, F2 and F3 ratios: permanent tooth pulp derived cell lines (14^{th} and 15^{th})

Mann-Whitney U Test was performed

- To compare the Fibroblastoid : Epithelioid cell ratio between the permanent and deciduous tooth pulp groups

Historical background

Attempts at cell culture began over hundred years ago when the German zoologist Wilhelm Roux showed that the neural plate from chicken embryos could be removed and maintained in warm saline solution for many days.[28] This concept was taken one step further by Ross Granville Harrison, an American born scientist and Yale professor who, in 1906 not only maintained amphibian nerve fibers *ex vivo*, but also developed conditions under which these nerve fibers were able to proliferate.[29] The groundwork laid down by Harrison was built upon by Nobel Prize winning scientist Alexis Carrel who was able to culture the heart of a chicken embryo for a period much longer than the normal lifespan of a chicken.[30]

For the next several years, it was mainly tissue explants that were used for experimentation. Harry Eagle, in 1955 demonstrated that the tissue extracts used to grow cells could be replaced with a synthetic and defined nutrient mixture containing amino acids, vitamins, carbohydrates, salts, and serum.[31] Taken together, these technologies paved the way for a whole new approach to scientific investigation using *in vitro* systems. But, perhaps the largest impact on society was the ability to use cell lines to grow purified viruses for vaccine production.

The next major advancement in the history of cell culture was in the 1970s with the development of hybridoma cell lines, which could be used for the production of monoclonal antibodies.[32] This technology

was developed by Cesar Milstein, Georges J. F. Köhler and Niels Kaj Jerne and resulted in an equally shared Nobel Prize in Medicine in 1984. In 1998, stem-cell research was catapulted forward by the work of James Thomson and John Gearhart who, independently of one another, grew human stem cells in culture.[32]

HeLa cells are a human epithelial cervical cancer, and the first human cells, from which a permanent cell line was established. On 9 February 1951, surgeon Lawrence Wharton Jr. removed the tissue from the patient Henrietta Lacks, a 31-year-old African American woman from Baltimore, in the Women's Clinic of the Johns Hopkins Hospital. The cells were from the carcinoma of the cervix and were expected to be examined for malignancy. The patient died eight months later from the disease. A portion of cells from the biopsy were sent to George Gey, the then head of the cell culture laboratory at Johns Hopkins Hospital. The cells were cultivated and propagated in cell culture so well that since then, they have been widely used in research. The HeLa cells were used in the establishment of the first polio vaccine by Jonas Salk. HeLa cells are now available in many laboratories of the world.[33]

Types of Stem Cells [34]

Although all stem cells share basic characteristics like the ability to perpetuate themselves and to generate mature cells by differentiation, they can be classified based on their ability to differentiate.

- **Totipotent:** The fertilized zygote, capable of independently giving rise to all embryonic and extra-embryonic tissues
- **Pleuripotent:** The inner cell mass of the blastocyst in the developing zygote and embryonic cells in culture, capable of giving rise to all embryonic cells and tissues
- **Multipotent fetal stem cells:** Cells derived from the three embryonic germ layers (ectoderm, mesoderm and endoderm) that become more committed to generating particular cells as organs and tissues are formed
- **Multipotent adult stem cells:** The cells that are thought to be tissue-specific and forming only one type of cell (unipotent)

Stem cells can also be classified based on origin

- Embryonic stem cells
- Adult stem cells

Embryonic stem cells:

Embryonic stem (ES) cells are totipotent cells, capable of differentiating into virtually any cell type, as well as being propagated indefinitely in an undifferentiated state.[35]

ES cells are derived from inner cell mass of the mammalian blastocysts that develop from zygotes that have been fertilized *in vitro*. ES cells can be maintained in culture as undifferentiated cell lines or induced to differentiate into many different lineages.

Sources of embryonic stem cells:

In vitro fertilization of embryos from the inner cell mass of blastocyst – Embryonic stem cells

Primordial germ cells from fetal gonads – Embryonic germ cells

Adult stem cells:

An adult stem cell is an undifferentiated (unspecialized) cell that is found in a differentiated (specialized) tissue. These cells have the ability to proliferate and this is referred to as long-term self-renewal. They can also give rise to mature cell types that have characteristic morphology and specialized function.

Sources of adult stem cells:

Pregnancy related tissue- umbilical cord, placenta and amniotic fluid.

Adult tissues- bone marrow, liver, epidermis, retina, brain, skeletal muscle, dental pulp and periodontal ligament.

Cadavers- Post-mortem human brain within 20 hours after death (cessation of vital functions including heartbeat, brain activity and breathing) – neural stem cells

Mesenchymal Stem Cells (MSC)

The concept of mesenchymal stem cells (MSCs, a term first coined by Arnold Caplan in 1991, can be traced to experiments demonstrating that transplantation of bone marrow (BM) to heterotopic anatomical sites resulting in *de novo* generation of ectopic bone and marrow.[36] These experiments were conducted with entire fragments of bone-free BM, so the exact identity of any cell functioning as a progenitor of differentiated bone cells could not be established. It was Friedenstein and co-workers, in a series of studies, [37] who demonstrated that the osteogenic potential was associated with a minor sub-population of BM cells. *In vivo* transplantation led to the recognition that multiple skeletal tissues (bone, cartilage, adipose tissue, and fibrous tissue) could be experimentally generated, *in vivo*, by the progeny of a single BM stromal cell.[37] Friedenstein and Owen called this cell an osteogenic stem cell or a BM stromal stem cell.[11] These cells were distinguishable from the majority of hematopoietic cells by their rapid adherence to tissue culture vessels and by the fibroblast-like appearance of their progeny in culture, pointing to their origin from the stromal compartment of BM.

While originally, the term MSCs specifically referred to cells in BM (bone marrow stromal cells, BMSCs), the current usage of the term has been extended to include cells from additional sources (synovium, adipose tissue, dental pulp, etc.) and from almost every postnatal connective tissue.

MSC can be isolated from different sources and they have been extensively characterized *in vitro* by the expression of markers like STRO-1, CD146 or CD44.[12] STRO-1 is a cell surface antigen used to identify osteogenic precursors in bone marrow, CD146 a pericyte marker, and CD44 a mesenchymal stem cell marker. MSC have a high self-renewal capacity and the potential to differentiate into mesodermal lineages forming cartilage, bone, adipose tissue, skeletal muscle and the stroma of connective tissues.[13]

Mesenchymal stem cells are also present in dental tissues. To date, five different human dental stem cells have been isolated and characterized: dental pulp stem cells, stem cells from exfoliated deciduous teeth, periodontal ligament stem cells, stem cells from apical papilla and dental follicle progenitor cells. These post-natal populations have mesenchymal-stem-cell-like qualities, including the capacity for self-renewal and multi-lineage differentiation.

Stem Cell Niche

The stem cell niche concept was first proposed as a specialized micro-environment needed for cells to retain their 'stemness'.[38] The niche is considered a fixed compartment of a three-dimensional structure containing elements that participate in the regulation of stem cell proliferation, controls the fate of stem cell progeny, and prevent the stem cells from exhaustion or death.[39, 40] The bone marrow micro-environment is a major site of MSC niche in the body. The DPSC niche in human dental pulp was identified by antibodies against STRO-1,

CD146, and pericyte-associated antigen (3G5) and was found to be localized in the perivascular and perineural sheath regions.[4] These STRO-1+/CD146+ DPSCs form a dentin-pulp-like complex *in vivo*. The STRO-1-positive region in the pulp of deciduous teeth is similar to that of permanent teeth, also in the perivascular regions. STRO-1/ CD146/CD44 staining of the PDL has shown that it is located mainly in the perivascular region, with small clusters of cells in the extravascular region, [41] suggesting that these are the niches of PDLSCs. STRO-1 staining of apical papilla has shown that the positive stain is located in the perivascular region as well as other regions scattered in the tissue. [38]

Therefore, in teeth, two different stem cell niches have been suggested: the cervical loop of rodent incisor for epithelial stem cells[42,43] and a perivascular niche in adult dental pulp for mesenchymal stem cells.[4]

Stem Cell Culture Medium [44]

Minimum Essential Medium (MEM), developed by Harry Eagle, is one of the most widely used of all synthetic cell culture media. Early attempts to cultivate normal mammalian fibroblasts and certain subtypes of HeLa cells revealed that they had specific nutritional requirements that could not be met by Eagle's Basal Medium (BME). MEM, which incorporates these modifications, includes higher concentrations of amino acids so that the medium more closely approximates the protein composition of mammalian cells. MEM has

been used for cultivation of a wide variety of cells grown in mono-layers. Optional supplementation of non-essential amino acids to the formulations that incorporate either Hanks' or Eagles' salts has broadened the usefulness of this medium. Minimum Essential Medium Eagle Alpha Modification or alpha-minimal essential medium (α-MEM) is the most enriched variation of the MEM formulation offered. It contains all 21 normal amino acids, some at increased concentrations. In addition, it contains 5 additional vitamins. α-modified minimal essential medium (α-MEM) with 2 mM glutamine and supplemented with 15% fetal bovine serum (FBS), 0.1 mM l-ascorbic acid phosphate, 100 U/ml penicillin, and 100 µg/ml streptomycin is used for the culture of dental pulp stem cells. Selection of a suitable lot of FBS is critical for successful MSC culture. FBS is selected on the basis of its colony-forming efficiency. Usually, a higher colony number is associated with better proliferation of MSCs.[27]

While dental pulp is a source of unidentified progenitors able to differentiate into odontoblast-like cells, Lopez-Cazaux *et al* [45] investigated the effect of two media; MEM (1.8mM Ca and 1mM) and RPMI 1640 (0.8mM Ca and 5mM) on the behaviour of human dental pulp cells. Their study indicated that MEM significantly increased cell proliferation and enhanced the proportion of α-smooth muscle actin positive cells, which represent a putative source of progenitors able to give rise to odontoblast-like cells.[45] In addition, MEM strongly stimulated alkaline phosphatase activity and was found to induce expression of transcripts encoding dentin sialophosphoprotein, an

odontoblastic marker, without affecting that of parathyroid hormone/parathyroid hormone related protein-receptor and osteonectin. This showed that not only proliferation but also differentiation into odontoblast-like cells was induced by rich calcium and poor phosphate medium (MEM) as compared to RPMI 1640.

Dental Pulp Cell Culture Methods

Studies have compared the growth of human pulp cells isolated by enzyme digestion/disaggregation and the outgrowth methods. Different isolation methods gave rise to different populations or lineages of pulp cells during *in vitro* passage. Cells isolated by enzyme digestion had a higher proliferation rate than those isolated by outgrowth.[46]

Huang *et al*, [46] Gronthos, Brahim *et al* [47] and Miura *et al* [48] followed the protocol described by Gronthos *et al* in 2000 [49] for the culture of dental pulp cells by the enzyme digestion method.[49]

According to Gronthos *et al*. (2000), [49] pulp tissue is digested in a solution of 2 mg/ml collagenase type I and 1 mg/ml dispase (Sigma, St. Louis, Mo., USA) for 30–60 minutes at 37°C. They then passed the digested tissues through a 70–μm cell strainer (Becton/ Dickinson, Franklin Lakes, N.J., USA) to give a cell suspension. They described the seeding of single cell suspensions (1×105 cells/flask) in 5×10 cm culture flasks containing α-minimum essential medium (α-MEM; Life Technologies/GIBCO BRL, Gaithersburg, Md., USA) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine,

100 µM L-ascorbic acid-2-phosphate, 100 U/ml penicillin-G, 100 µg/ml streptomycin, and maintained under 5% CO_2 at 37°C. [49]

Huang *et al*. (2006)[46] performed the outgrowth method where they placed pulp tissue explants (2×2×1 mm fragments) in 6-well plates with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics. When the cells reached confluence, they transferred the cells to 5×10 cm culture flasks and continuously passaged them when they became confluent.[46]

The advantage of culturing pulp cells via the outgrowth method, according to Huang *et al,* [46] was convenience and ease of culture, although more time was needed to allow sufficient numbers of cells to migrate out of the tissue (up to 2 weeks). They found that the digestion method released all cells from the tissue but the process was technically difficult and some degree of cell damage and cell loss occurred.[46] Gronthos, Brahim *et al*. in 2002 [47] reported that with enzyme disaggregation method, colony formation was seen and they further characterized these colonies to study their stem cell properties.[47]

Cell Culture - Limitations and Problems

Cell culture is referred to as an *ex vivo* study of the cellular milieu. The cell is not in its normal physiological and original environment. Cell culture is simply an attempt to provide a simulated environment.[44]

The limitations of cell culture that have been enlisted include the finite doubling potential of most normal cells, the possibilities for unexpected infection with microorganisms and cross-contamination with other cell types. Takeda *et al* (2008)[50] reported the loss of nine of their DPSC cultures to bacterial/ fungal contamination. Some cultured cells have been reported to have a tendency to change their morphology, functions, or the range of genes they express.[51]

There are several common problems encountered when culturing cells. One of the major problems is the rapid pH shift in the medium which is usually caused by incorrect carbon dioxide (CO_2) tension in the incubator. This can be corrected by increasing or decreasing the percentage of carbon dioxide in the incubator based on the concentration of sodium bicarbonate in the culture medium. Bacterial or fungal contamination and insufficient bicarbonate buffering have also been implicated in causing a shift in the pH of the medium. [44]

Trypsinizing the cells for an extended period of time is said to cause poor adherence of the cells to the culture vessel. [44]

A decrease in the growth of cells in culture is said to be caused due to a change in medium or serum; depletion, absence, or breakdown of essential growth-promoting components such as L-glutamine or growth factors; or a low-level bacterial or fungal contamination.

Death of the cells is attributed to the absence of carbon dioxide or temperature fluctuations in the incubator. [44]

Mesenchymal / Stromal Stem Cell Markers

STRO-1: The murine IgM monoclonal antibody, STRO-1, produced from an immunization with a population of human CD34+ bone marrow cells, can identify a cell surface antigen expressed by stromal elements in human bone marrow. A STRO-1 enriched subset of marrow cells is capable of differentiating into multiple mesenchymal lineages including hematopoiesis-supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts and chondrocytes. STRO-1 is a useful antibody for the identification, isolation and functional characterization of human bone marrow stromal cell precursors. [14]

CD44: CD44 is a receptor for hyaluronic acid and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases. Its function is controlled by its post-translational modifications.CD44 glycosylation also controls its binding capacity to fibrin and immobilized fibrinogen. A specialized sialofucosylated glycoform of CD44 called H-CELL is found natively on human hematopoietic stem cells, and is a highly potent E-selectin and L-selectin ligand. H-CELL functions as a "bone homing receptor", directing migration of human hematopoietic stem cells and mesenchymal stem cells to bone marrow. Variations in CD44 have been used as cell surface markers for some breast and prostate cancer stem cells.[4, 9, 10, 14, 38, 47, 51]

CD146: CD146 is also known as the melanoma cell adhesion molecule (MCAM). It is a 113 kDa cell adhesion molecule used as a marker for mesenchymal and endothelial cell lineage. In humans, the CD146 protein is encoded by the MCAM gene. Its function is not completely understood, but evidence points to it being part of the endothelial junction associated with the actin cytoskeleton. It is a member of the Immunoglobulin superfamily and consists of five Ig domains, a transmembrane domain, and a cytoplasmic region. It is expressed on chicken embryonic spleen and thymus, activated human T cells, endothelial progenitors like angioblasts and mesenchymal stem cells. It is s strongly expressed on blood vessel endothelium and smooth muscle.

CD146 has been used as a marker for mesenchymal stem cells isolated from multiple adult and fetal organs. [4, 9, 10, 14, 38, 47, 51, 52]

Stem Cells from Dental Pulp

The identification and isolation of an odontogenic progenitor population in adult dental pulp was first reported by Gronthos and co-workers in 2000.[49] This group described the identification of dental pulp stem cells (DPSCs) by their rapid proliferation rates and ability to form mineralized tissues both *in vitro* and *in vivo*. They suggested the existence of stem cell niches within the dental pulp which showed cellular differentiation and multipotentiality.

The cellular characteristics of these DPSCs were compared with those of bone marrow stem cells. Both dental pulp and bone marrow

stem cell populations expressed similar stem cell surface markers- CD44, CD106, CD146, and STRO-1. [4, 9, 10, 14, 38, 47, 51, 52]

Subsequent studies [46, 47, 48] isolated single-cell colony derived populations of DPSCs which demonstrated multi-potentiality. They were able to form adipocytes and neural precursors *in vitro*, in addition to dentin-like tissue following transplantation into immunocompromised mice.

In contrast to bone marrow stem cells, DPSCs showed a 30% higher proliferation rate and a higher growth potential. This higher rate of proliferation has been linked to the increased pulp cell expression of specific cell cycling mediators, namely cyclin-dependant kinase 6 and insulin-like growth factor.[49]

Transplantation of DPSCs into immunocompromised mice resulted in the formation of a dentine-like tissue, whereas bone marrow stem cells produced a tissue resembling that of lamellar bone. This suggests that inherently different regulatory mechanisms exist within the two stem cell populations. [49]

Attempts were made to isolate and characterize progenitor/stem cell populations from adult dental pulp, with the intention of achieving a more defined clonal population of cells. A mesenchymal stem progenitor population expressing the cell surface receptor STRO-1 was isolated from adult dental pulp.[4] The isolation strategy was similar to that previously used for the isolation of bone marrow stem cells. [4]

These STRO-1 positive cells were found to differentiate down neurogenic, adipogenic, myogenic and chondrogenic lineages, and made a mineralized matrix when cultured in 'odontogenic'-inducing conditions. When cells isolated for STRO-1 were compared with those which were negative for this mesenchymal stem cell marker, only STRO-1 positive cells were capable of differentiating into odontoblast-like cells, indicating the importance of these cells in dentine repair processes. [49]

Miura *et al*. in 2003, [48] provided evidence that the remnant dental pulp from exfoliated deciduous teeth contained a multipotent stem-cell population. They showed that these stem cells could be isolated and expanded *ex vivo*. Previous experiments had shown that the dental pulp tissue of adult teeth contained a population of DPSCs that were capable of differentiating into odontoblasts and adipocytes and forming a dentin pulp-like complex after *in vivo* transplantation. [49]

Miura *et al*. [48] believed that SHED are distinct from DPSCs with respect to their higher proliferation rate, increased cell-population doublings, sphere-like cell-cluster formation, osteo-inductive capacity in vivo and failure to reconstitute a dentin–pulp-like complex. SHED was said to represent a population of multipotent stem cells that are more immature than previously examined postnatal stromal stem-cell populations.

Suchanek *et al.* [53] isolated stem cells from permanent and deciduous teeth and compared their growth and phenotypes. They were able to cultivate SHED over 45 population doublings. In their experiments, they found SHED to have longer population doubling time as compared to DPSCs. The SHED proliferation rate was 50% slower. SHED also comprised of more 'rounded cells without long processes'.[53]

Phenotypic Characterisation / Phonotype Models

Studies on rat skin and lung fibroblasts revealed the presence of three sub-populations of cells-FI, FII, FIII based on their morphologies and proliferation.[24] FI cells were spindle shaped and showed high proliferation potential. FII cells were "epithelioid" and proliferated at a slower rate as compared to FI cells. FIII fibroblasts were large, stellate cells that proliferated slower than the other types. [24]

Growth Characterization and Population Doubling Time (PDT)

In vitro studies on skin fibroblast populations have indicated they undergo cumulative population doublings.[25,26] When the growth capacity of the mitotic fibroblast got exhausted, they differentiated spontaneously into post-mitotic fibroblast populations. Mitotic fibroblast were characterized as F-I, F-II, F-III and post-mitotic as F-IV, F-V, F-VI, F-VII. The F-I cell was a small spindle shaped cell. F-II was a small "epithelioid"[24,25] cell. F-III was a large pleomorphic, epithelioid cell. F-IV was a large spindle shaped cell. F-V was a larger

epithelioid cell. F-VI, the largest epitheloid cell of the fibroblast series and F-VII was a degenerating fibroblast.[25]

A growth curve for a cell line shows a *lag phase*, which is the time taken for the cells to adapt to the new environment. *Log phase* is the phase when cells start to divide and increase in number exponentially. The growth curve then reaches a *plateau phase* when the cells the reach confluence. [23, 26]

The slope of the growth curve in the log phase yields the population doubling time (PDT). PDT derived from the growth curve is not equivalent to cell cycle time or cell generation time. PDT is an average value from the whole population of cells which includes dividing cells, non-dividing cells and dying cells, so PDT can be influenced by non-dividing and dying cells as well. [23]

Applications of Stem Cells [18, 34, 38, 54, 55, 56]

A stem cell is a cell that can continuously produce unaltered daughters and also generate cells with different and more restricted properties. Stem cells can divide either symmetrically (allowing the increase of stem cell number) or asymmetrically. Asymmetric divisions keep the number of stem cells unaltered and are responsible for the generation of cells with different properties. These cells can either multiply (progenitors or transient amplifying cells) or be committed to terminal differentiation. Progenitors and transient amplifying cells have a limited lifespan and can only reconstitute a tissue for a short period of time when transplanted. On the other hand, stem cells are

self-renewing and therefore, can generate any tissue for a lifetime. This is a key property for successful therapy. The capacity to expand stem cells in culture is an important step for regenerative medicine, and a considerable effort has been made to evaluate the consequences of the cultivation on stem cell behaviour.

Applications in Dentistry

(i) Basic dental research:

To characterize the functional role of differentially expressed genes.

(ii) Clinical dental research:

- To repair damaged tooth structure – endodontic therapy.
- Induce bone regeneration.
- To treat neural tissue injury or degenerative diseases.

(iii) Periodontal ligament regeneration

(iv) Dentin regeneration

(v) Tissue engineering

Studies on dental epithelial histomorphogenesis have confirmed the role of the cap-stage mesenchyme in the control of tooth morphogenesis and also shown that the mesenchyme can induce disorganized epithelial cells to restore a complete histogenesis of the enamel organ. [54] This epithelial cell plasticity (i.e., their ability to undergo conversion between different epithelial cell types) is a pre-requisite for enamel tissue engineering and paves the way for bio-engineering of the human tooth.

Tissue engineering studies have been carried out on animal models where MSCs were isolated from rat bone marrow and induced to differentiate into chondrogenic and osteogenic cells, *in vitro*. These cells were then loaded onto human mandibular condyle-shaped polyurethane molds and implanted into immunodeficient mice. Eight weeks after the *in vivo* implantation of the osteochondral constructs, mandibular condyles were formed. [21]

Studies on animal models have also proven the feasibility of bio-engineering teeth. Rat tooth-bud cells when cultured, seeded onto bio-degradable scaffolds and implanted into the jaws of adult rat hosts, formed small, organized, bioengineered tooth crowns containing dentin, enamel, pulp, and periodontal ligament tissues. Radiographic, histological, and immunohistochemical analyses showed that the bioengineered teeth consisted of organized dentin, enamel, and pulp tissues. [55]

Bone marrow stem cells (BMSCs) have the potential to re-create tissues of the craniofacial region. *Ex vivo* expanded BMSCs with scaffolds have been used to aid in the re-building of the hard structures of the face. [56]

Taken together, these recent findings indicate that the control of morphogenesis and cyto-differentiation is a challenge that requires an understanding of the cellular and molecular events involved in the development, repair and regeneration of teeth. The identification of mesenchymal stem cells in the tooth and the knowledge of molecules

involved in stem cell fate is a significant achievement. *In vitro* and *in vivo* experiments using these cells have shown promising results. However, scientific knowledge alone is not enough and the main challenge in stem-cell therapy is to find a compromise between the scientific research and clinical application that benefits the patient.

The present study was done to standardize the procedure for the isolation and culture of mesenchymal stem cells from the dental pulp of permanent and deciduous teeth. Fifteen permanent teeth and thirteen deciduous teeth from twenty-five subjects were used. The enzymes (collagenase, dispase) and their amounts, the duration of enzyme disaggregation, level of carbon dioxide and pH of the culture medium were altered with each tooth under study to arrive at a protocol for the culture of the cells from these teeth. [Master Chart- Annexure II]

We studied the growth characteristics and phenotype of the cultured DPSC and SHED. We studied the colony forming efficiency of the cultured DPSC. The growth pattern of the mesenchymal stem cells from permanent teeth and exfoliated deciduous teeth was studied by inoculating the cells from the fourth passage on 24-well plates. The growth curve was plotted and population doubling time (PDT) derived from the exponential growth phase.[26] The phenotypes of the fibroblastoid cell sub-populations was studied by classifying them as F_1, F2 and F_3 fibroblastoid cells based on the description given by Mollenhauer and Bayreuther in 1986 on rat skin fibroblasts.[24] The colony forming efficiency of the DPSCs was evaluated using fifth passage cells from the permanent tooth pulp.[27]

In order to compare the phenotypic characteristics of the mesnchymal stem cells from permanent and deciduous teeth, ten cells were randomly counted from day 6, for fifteen consecutive days and classified as fibroblastoid or epithelioid based on their morphology.

Fifteen permanent teeth (numbered 1st, 2nd....15th permanent tooth pulp culture) and thirteen deciduous teeth (numbered 1st, 2nd...10th deciduous tooth pulp culture) from twenty-five subjects formed the study group. The protocol followed for each tooth was as follows [Ref. Master Chart- Appendix II, Graph 1 and Graph 2]

1st permanent tooth pulp culture

Pulp obtained from a third molar was disaggregated for **60 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). No cells were seen on day 3, after plating the cells. On day 6, granular material was observed throughout the medium in the cell culture plate. All cell culture glassware and plastics were thoroughly washed and autoclaved.

2nd permanent tooth pulp culture

Pulp obtained from a maxillary third molar was disaggregated for **45 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). No cells were seen on day 3, after plating the cells. On day 6, granular material was observed through out the medium in the cell culture plate. A smear was made of the precipitate after centrifugation of the contaminated cell culture medium. Microscopic examination of the Haematoxylin and Eosin stained smear showed Gram negative Cocci. When sent to the laboratory for speciation, the results were negative for the presence of microorganisms. All cell culture glassware and plastics were

thoroughly washed, wrapped in aluminium foil and autoclaved. Caps of the vials were wiped with spirit and flamed before use.

3rd permanent tooth pulp culture

Pulp obtained from a maxillary third molar was disaggregated for **60 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). No cells were seen on day 3 and day 6, after plating the cells. The level of carbon dioxide in the cylinder was falling and the cells had to be discarded after the carbon dioxide cylinder was empty on day 9.

4th permanent tooth pulp culture

Pulp obtained from a 23year old male patient's mandibular third molar was disaggregated for **18 hours** in enzyme solution (collagenase-3mg in 1ml Dulbecco's Phosphate Buffered Saline). No cells were seen on day 3, after plating the cells. On day 6, granular material was observed through out the medium in the cell culture plate. A smear was made of the precipitate after centrifugation of the contaminated cell culture medium. Microscopic examination of the Haematoxylin and eosin stained smear showed Gram negative Cocci. When sent to the laboratory for speciation, the results were negative for the presence of microorganisms. All cell culture glassware and plastics were thoroughly washed and autoclaved. Caps of the vials were wiped with spirit and flamed before use.

5th permanent tooth pulp culture

Pulp obtained from a maxillary third molar was disaggregated for **40 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 3, one cell cluster with flat, epithelioid cells and few spindle shaped fibroblastoid cells as outgrowths were seen. The level of carbon dioxide in the cylinder was falling and the cells had to be discarded after the carbon dioxide cylinder was empty on day 6.

6th permanent tooth pulp culture

Pulp obtained from a mandibular third molar was disaggregated for **30 minutes** in enzyme solution (collagenas-2mg and dispase 1mg, in 1ml Dulbecco's Phosphate Buffered Saline).On day 3, after plating the cells, two cell clusters with flat, epithelioid cells and few spindle shaped fibroblastoid cells as outgrowths were seen. On day 6, granular material was observed through out the medium in the cell culture plate. A smear was made of the precipitate after centrifugation of the contaminated cell culture medium. Microscopic examination of the Haematoxylin and eosin stained smear showed Gram negative Cocci. When sent to the laboratory for speciation, the results were negative for the presence of microorganisms. All cell culture glassware and plastics were thoroughly washed and autoclaved. Caps of the vials were wiped with spirit and flamed before use. The use of cotton wool was discontinued and autoclaved gauze used for wiping the lamina flow and instruments. All head caps and mouth-masks were washed and

autoclaved everyday and the use disposable head caps and mouth-masks discontinued.

7th, 8th and 9th permanent tooth pulp cultures

Cells were discarded after days 9 and 12 because they were contaminated with granular, black bodies.

10th permanent tooth pulp culture

Pulp obtained from a mandibular third molar was disaggregated for **60 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 6, three cell clusters with flat, epithelioid cells and few spindle shaped fibroblastoid cells as outgrowths were seen. On days 6, 9 and 12, the number of spindle-shaped, fibroblastoid cells appeared fewer in number. The flat, epithelioid cells did not increase in number and the cells did not reach confluency.

11th and 12th permanent tooth pulp cultures

Pulp obtained from a mandibular second premolar and third molar was disaggregated for **17 and 16 hours** respectively in enzyme solution (crude collagenase-3mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 6, three cell clusters with flat, epithelioid cells and few spindle shaped fibroblastoid cells as outgrowths were seen. On days 9, 12 and 18, the number of spindle-shaped, fibroblastoid cells appeared fewer in number. The flat, epithelioid cells did not increase in number and the cells did not reach confluency.

13th permanent tooth pulp culture

Pulp obtained from a third molar was disaggregated for **17 hours** in enzyme solution (crude collagenase-3mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 9, five cell clusters with flat, epithelioid cells and spindle-shaped fibroblastoid cells as outgrowths were seen. The cells reached confluency after forty-five days and were predominantly flat, epithelioid cells. Trypsinisation was done on day 46.

14th permanent tooth pulp culture

Pulp obtained from a mandibular third molar was disaggregated for **60 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 3, four to five cell clusters with flat, epithelioid cells and spindle shaped fibroblastoid cells as outgrowths were seen. On days 6, 9 and 12, the cells were seen to be predominantly spindle-shaped and fibroblastoid in morphology. The cells reached confluency on day 10 and trypsinisation done on day 11. The cells from the fourth sub-culture were used to determine the growth curve and perform the sub-population analysis.

15th permanent tooth pulp culture

Pulp obtained from a mandibular third molar was disaggregated for **18 hours** in enzyme solution (crude collagenase-3mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 3, seven cell clusters with flat, epithelioid cells and spindle shaped fibroblastoid cells as outgrowths were seen. On days 6, 9 and 12, the cells were seen to be

predominantly spindle-shaped and fibroblastoid in morphology and increasing in number. The cells reached confluency on day 15 and trypsinisation was done on day 16. The cells from the fourth sub-culture were used to study growth curve and sub-population analysis.

1st 2nd and 3rd deciduous tooth pulp cultures

The amount of pulp tissue obtained from mandibular canine, mandibular molars and mandibular canine respectively, was insufficient to carry out tissue culture.

4th deciduous tooth pulp culture

Pulp obtained from the mandibular canine of a ten year subject was cultured as an explant in 40% Fetal Bovine Serum, without subjecting the tissue to enzymatic disaggregation. On day 3, fungal hyphae and spore forms were seen in the cell culture medium. The plate was discarded.

5th deciduous tooth pulp culture

Pulp obtained from the deciduous mandibular central incisors was disaggregated for **40 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). No cells were seen on days 3, 6, 8, 12 and 18 after plating the cells. The plate was discarded.

6th deciduous tooth pulp culture

Pulp obtained from the a mandibular central incisor was disaggregated for **45 minutes** in enzyme solution (collagenase-2mg and

dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). No cells were seen on day 3, after plating the cells. On day 6, granular material was observed through out the medium in the cell culture plate. All cell culture glassware and plastics were thoroughly washed and autoclaved.

7th deciduous tooth pulp culture

Pulp obtained from a mandibular central incisor was disaggregated for **45 minutes** in enzyme solution (collagenase-2mg and dispase 1mg in 1ml Dulbecco's Phosphate Buffered Saline). No cells were seen on days 3, 6, 8, 12 and 18 after plating the cells. The plate was discarded.

8th deciduous tooth pulp culture

Pulp obtained from a mandibular canine was disaggregated for **16 hours** in enzyme solution (crude collagenase-3mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 3, three cell clusters with flat, epithelioid cells and few spindle shaped fibroblastoid cells as outgrowths were seen. On days 6, 9 and 12, the number of spindle-shaped, fibroblastoid cells appeared fewer in number. The flat, epithelioid cells did not increase in number and the cells did not reach confluency.

9th deciduous tooth pulp culture

Pulp obtained from a mandibular canine was disaggregated for **80 minutes** in enzyme solution (collagenase-2mg and dispase-1mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 3, three cell clusters with flat, epithelioid cells and few spindle shaped fibroblastoid cells as

outgrowths were seen. On days 6, 9 and 12, the number of spindle-shaped, fibroblastoid cells appeared fewer in number. The flat, epithelioid cells did not increase in number and the cells did not reach confluency.

10th deciduous tooth pulp culture

Pulp obtained from two mandibular canines was disaggregated for **5 hours** in enzyme solution (crude collagenase-3mg in 1ml Dulbecco's Phosphate Buffered Saline). On day 3, four to five cell clusters with spindle shaped fibroblastoid cells and a few flat, epithelioid cells as outgrowths were seen. On days 6, 9 and 12, the cells were seen to be predominantly spindle-shaped and fibroblastoid in morphology.

F1:F2:F3 Fibroblastoid cell sub-population ratio of DPSC from the fourth passage of 14th permanent pulp (Graph 3, Table 1, 2)

There was a change in the ratio of F1, F2 and F3 phenotypes from the first through the eighth day of culture.

This was due to a relative decrease in the F1 (p= 0.74) and F2 (p=0.001) sub-populations and a relative increase in the F3 (p=0.001) sub-population over the eight day period.

However, only the decrease in the F2 sub-population of cells and increase in the F3 sub-population were statistically significant. (p=0.001)

F1:F2:F3 Fibroblastoid cell sub-population ratio of DPSC from the fourth passage of 15th permanent pulp (Graph 4, Table 3, 4)

There was a change in the ratio of F1, F2 and F3 phenotypes from the first through the eighth day of culture.

This was due to a relative decrease in the F1 (p=0.026) sub-population and a relative increase in the F3 (p=0.005) and F2 (p=0.778) sub-populations over the eight day period.

However, only the decrease in the F1 sub-population of cells and increase in the F3 sub-population was statistically significant. (p=0.005 and p=0.778 respectively)

Comparison of phenotypes of cells in first passages of 14th permanent pulp and 10th deciduous tooth pulp - (Table 5)

The cells from the permanent tooth pulp showed a higher proportion of spindle shaped fibroblastoid cells whereas a higher proportion of epithelioid cells were seen in the deciduous pulp culture. The difference was statistically significant at 5% level, $p<0.05$.

Growth curve of DPSC obtained from the fourth passage of 14th permanent tooth pulp- (Graph 5, Table 6)

The cells were seeded in the concentration of 12 x10^3 cells/ well/ ml. There was a loss of cells within 12 hours of attachment time. The seeding efficiency was 88.9%. There was a steady increase in the slope of the growth curve from day 1 to day 6. Population doubling time calculated from the slope of the graph was 26 hours. Plateau phase was reached by the 7th day of the culture.

Growth curve of DPSC obtained from the fourth passage of 15th permanent tooth pulp- (Graph 6, Table 7)

The initial seeding concentration was 12 x10^3 cells/ well/ ml. A loss of cells within 12 hours of attachment time was seen. The seeding efficiency was 91.7%. A steady increase in the slope of the growth curve from day 1 to day 8 was observed. Population doubling time calculated from the slope of the graph was 27 hours. The cells reached a plateau phase by the 7th day of culture.

Growth curve of DPSC obtained from the third passage of 10th deciduous tooth pulp- (Graph 7, Table 8)

The initial seeding concentration was 12 x10^{3} cells/ well/ ml. A loss of cells within 12 hours of attachment time was seen. The seeding efficiency was 84.25 %. A steady increase in the slope of the growth curve from day 1 to day 8 was observed. Population doubling time calculated from the slope of the graph was 22 hours. The cells reached a plateau phase by the 7th day of culture

Colony forming efficiency of DPSC derived from the fifth passage of 14th permanent pulp (Table 9)

Clonal Assay Plate I- 10 colonies were observed. The colony forming efficiency was calculated to be 25%

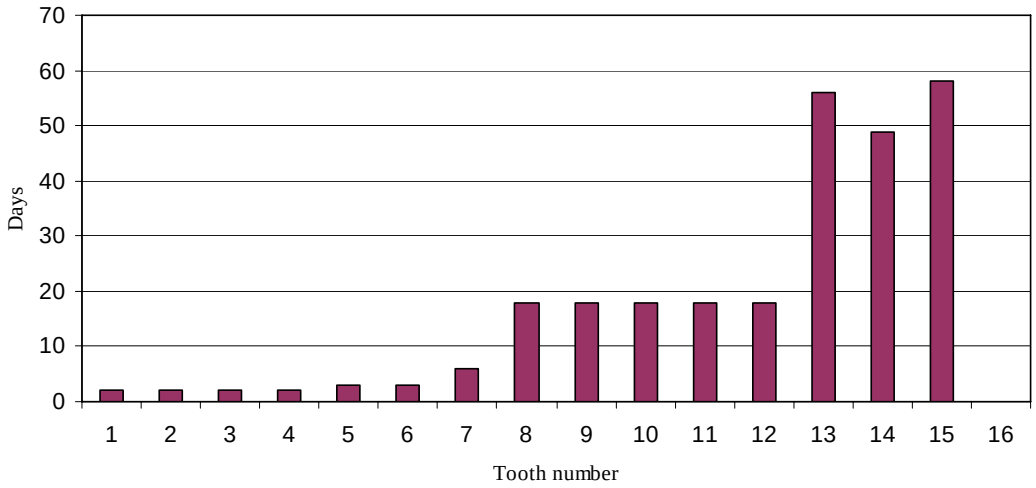
Clonal Assay Plate II- 7 colonies were observed. The colony forming efficiency was calculated to be 16.5%

Clonal Assay Plate III- 8 colonies were observed. The colony forming efficiency was calculated to be 17.1%.

Therefore, an average of 17.1% DPSCs derived from the 14th permanent pulp were capable of forming colonies

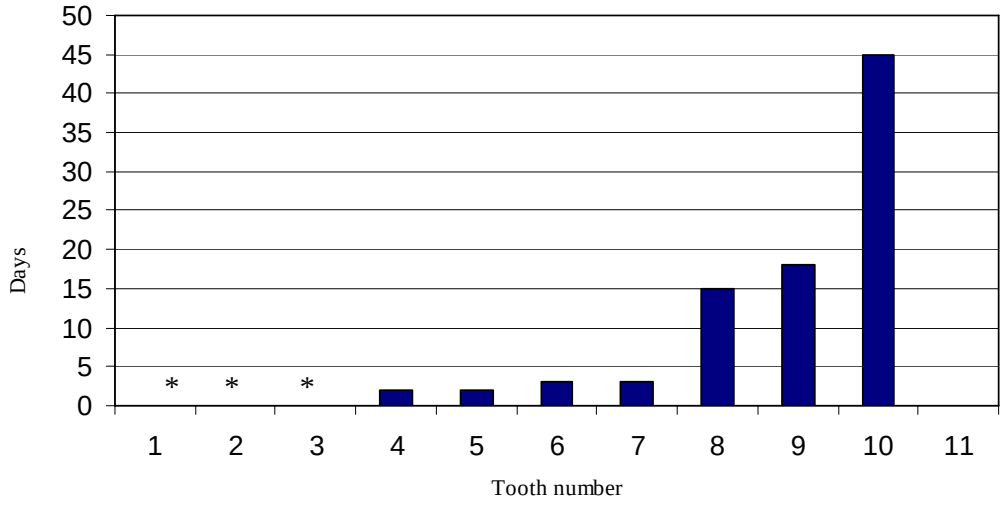
Graph 1

PERMANENT TEETH -CULTURE PERIOD



Graph 2

DECIDUOUS TEETH -CULTURE PERIOD



* Cells of sufficient number to propagate culture were not obtained

Graph 3

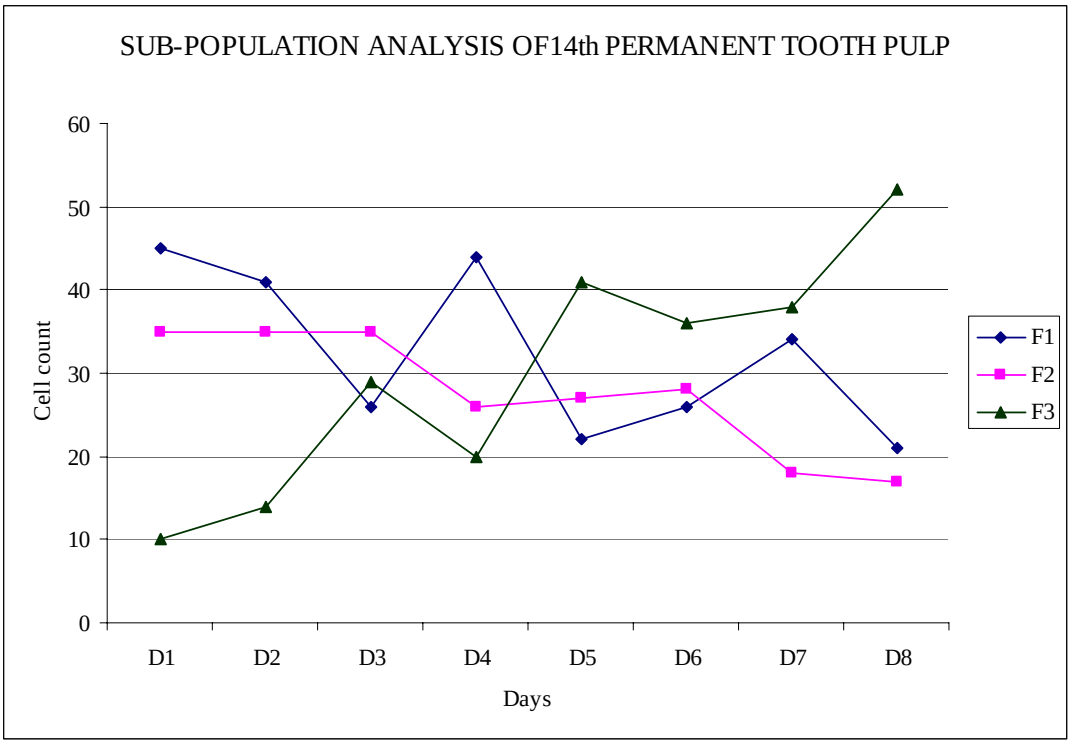


Table 1
Sub-population proportion in 90 cells for 8 days

DAYS	F1	F2	F3
1	45	35	10
2	41	35	14
3	26	35	29
4	44	26	20
5	22	27	41
6	26	28	36
7	34	18	38
8	21	17	52

Table 2
Correlation coefficient of the sub-populations

Sub population	Correlation coefficient (r)
F1	-0.6609
F2	-0.9242 *
F3	0.9201 *

* Statistically significant at 5% level, $p<0.05$

Graph 4

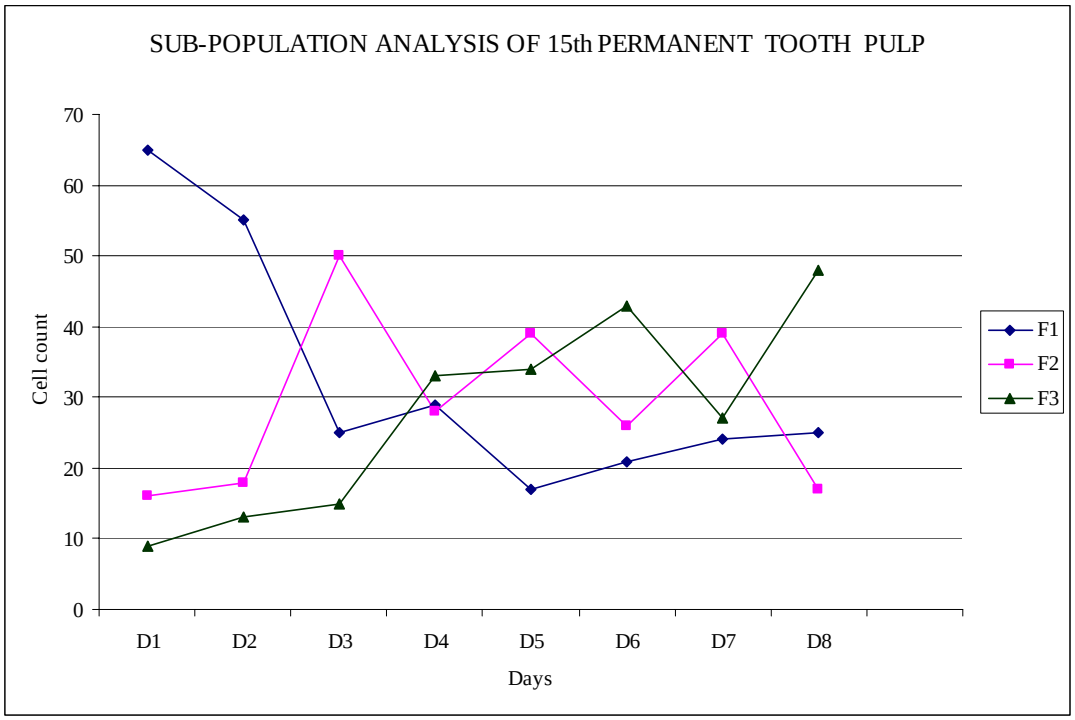


Table3
Sub-population proportion in 90 cells for 8 days

DAYS	F1	F2	F3
1	65	16	9
2	55	18	13
3	25	50	15
4	29	28	33
5	17	39	34
6	21	26	43
7	24	39	27
8	25	17	48

Table 4
Correlation coefficient of the sub-populations

Sub population	Correlation coefficient (r)
F1	-0.7670*
F2	0.1195
F3	0.8695*

*statistically significant at 5% level, $p<0.05$

Table 5

Fibroblastoid: Epithelioid cells in permanent and deciduous tooth pulp cultures counted over 15 consecutive days

	Permanent Teeth *		Deciduous Teeth *	
	Fibroblastoid cells	Epithelioid cells	Fibroblastoid cells	Epithelioid cells
Day 6	10	0	6	4
Day7	10	0	8	2
Day 8	10	0	7	3
Day 9	9	1	6	4
Day 10	9	1	6	4
Day 11	9	1	6	4
Day 12	8	2	7	3
Day13	9	1	6	4
Day 14	8	2	5	5
Day 15	7	3	6	4
Day 16	7	3	6	4
Day 17	7	3	5	5
Day 18	6	4	6	4
Day 19	7	3	5	5
Day 20	6	4	5	5

* Statistically significant at 5% level, $p<0.05$

Graph 5

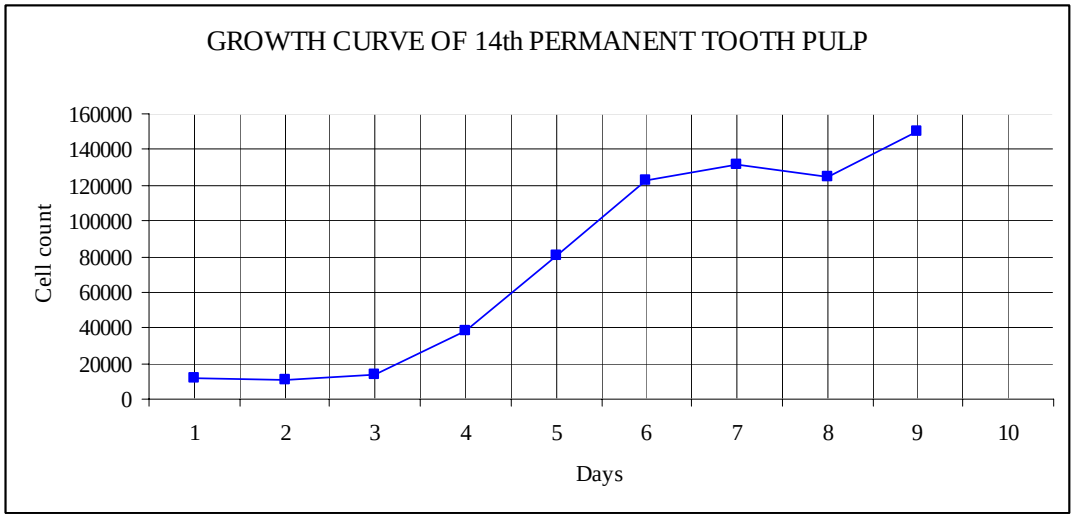


Table 6

Days	Cells/Well/ml x10^3	Slope	SE	Population Doubling Time (hours)	Seeding efficiency (%)
0	12.000	27.870	19.32	26	88.89
1	10.667				
2	13.700				
3	38.110				
4	80.100				
5	123.110				
6	131.710				
7	124.200				
8	150.440				

SE= standard error

Graph 6

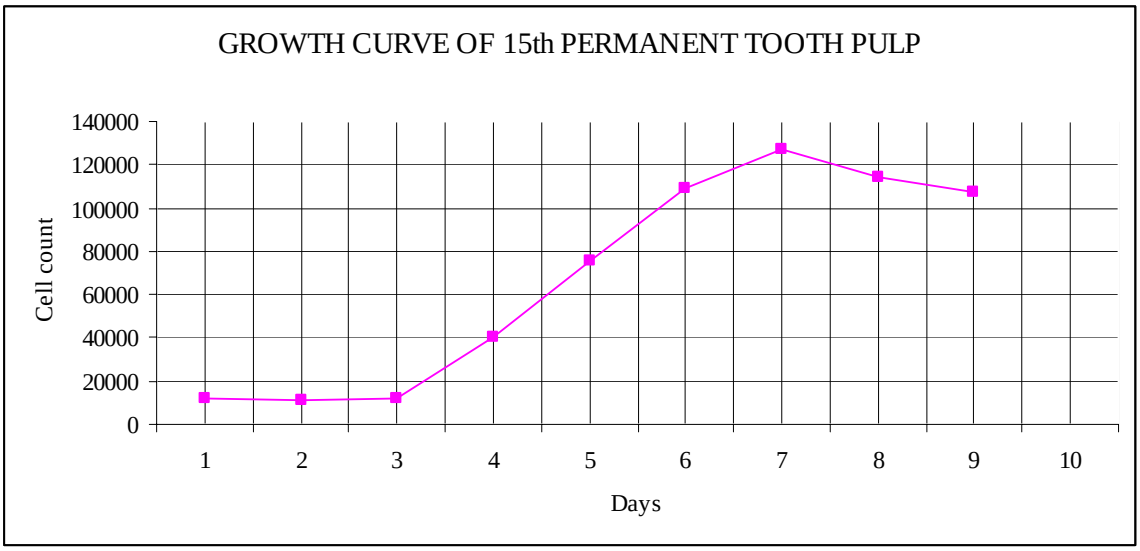


Table 7

Days	Cells/Well/ml x10^3	Slope	SE	Population Doubling Time (hours)	Seeding efficiency (%)
0	12.000	21.757	16.32	27	91.66
1	11.000				
2	12.000				
3	40.444				
4	75.777				
5	109.000				
6	126.778				
7	114.440				
8	107.333				

SE= standard error

Graph 7

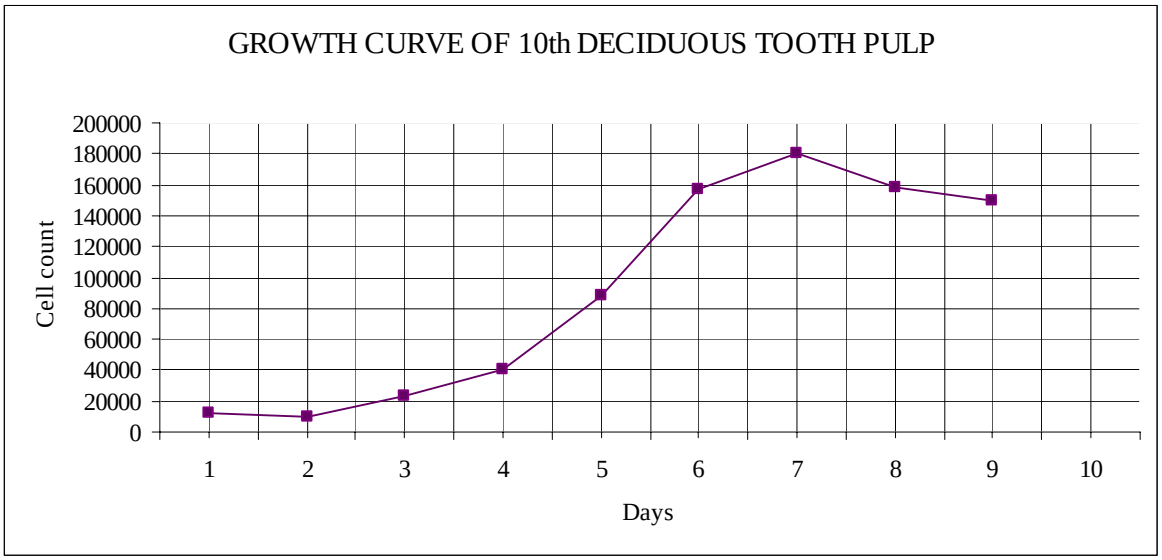
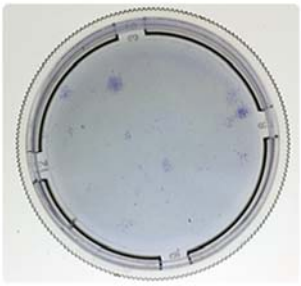


Table 8

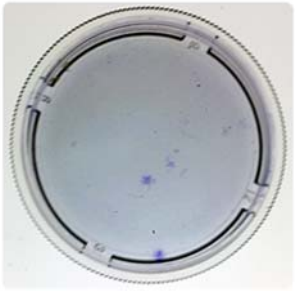
Days	Cells/Well/ml x10^3	Slope	SE	Population Doubling Time (hours)	Seeding efficiency (%)
0	12.000	30.887	25.11	22	84.25
1	10.110				
2	22.770				
3	41.000				
4	88.330				
5	157.440				
6	180.110				
7	158.000				
8	150.000				

SE= standard error

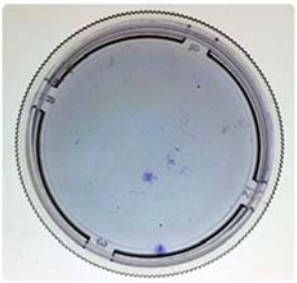
Estimation of colony forming efficiency



Clonal assay I



Clonal assay II



Clonal assay III

Table 9

Clonal Assay	Number of Colonies	Colony forming efficiency
I	10	25
II	7	16.5
III	8	20

Clonal Assay I - 10 colonies counted, colony forming efficiency = 25%

Clonal assay II- 7 colonies counted, colony forming efficiency = 16.5%

Clonal assay III- 8 colonies counted, colony forming efficiency = 20%

Therefore, an average of 17.1% of cells derived from permanent tooth pulp were capable of forming colonies

Figure1. Carbon dioxide incubator

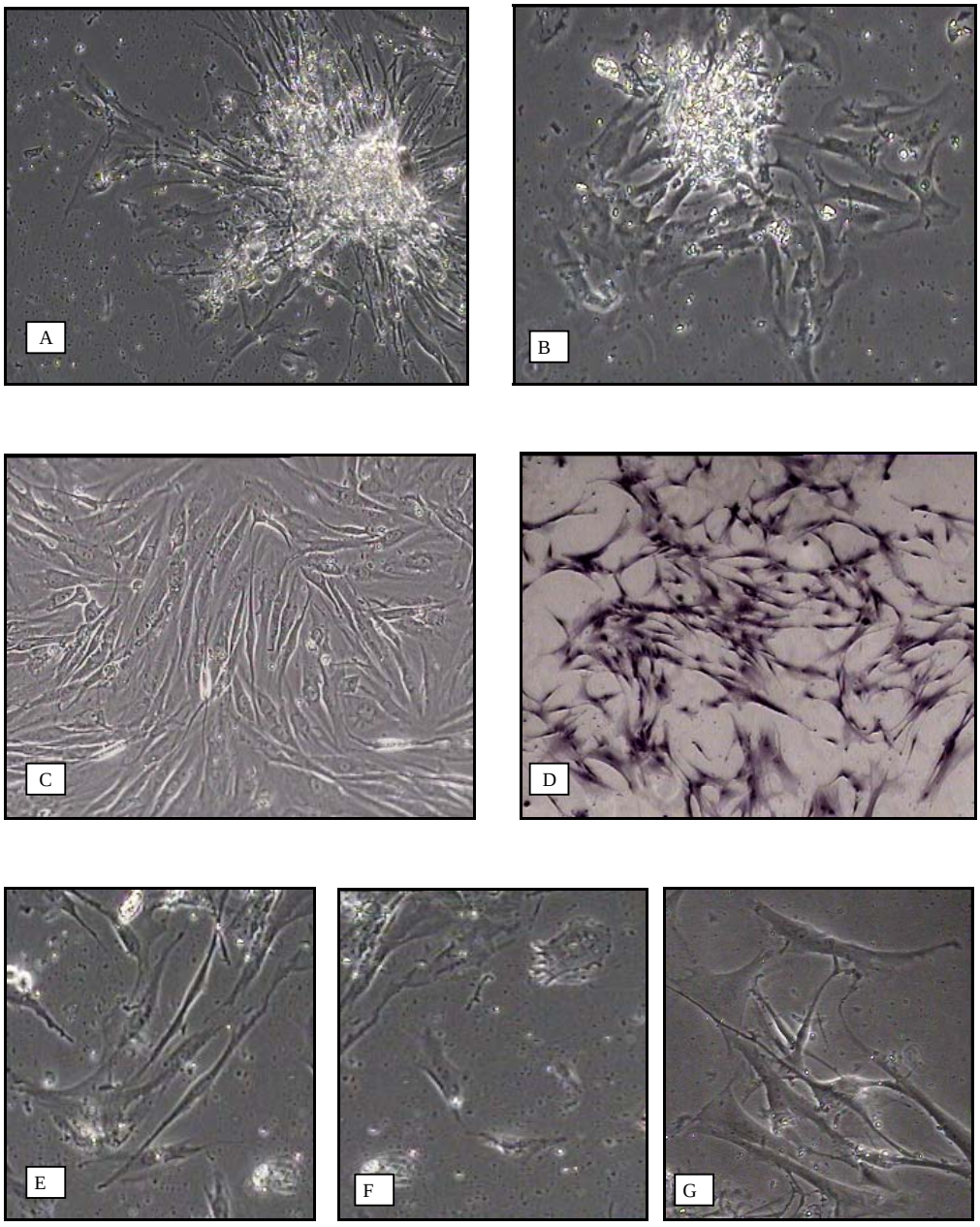
Figure2. Laminar flow

Figure3. Armamentarium for isolation of pulp from tooth

Figure4. Cell culture armamentarium

Figure5. Cell culture reagents

Figure 6. Mesenchymal stem cells from dental pulp



A - Cell outgrowth- Fibroblastoid cells, **B** - Cell outgrowth- Epithelioid cells

C - Cells in confluency , **D** - Cells after fixing and staining with haematoxylin and eosin

E - F1 phenotype, **F** - F2 phenotype, **G** - F3 phenotype

During tooth formation, interaction between epithelial and dental papilla cells promotes tooth morphogenesis by stimulating a sub-population of mesenchymal cells to differentiate into odontoblasts, which in turn form primary dentin. After tooth eruption, reparative dentin is formed by odontoblasts in response to abrasion, erosion or dentinal damage caused by bacteria. These odontoblasts are thought to arise from the proliferation and differentiation of a precursor population, residing within the pulp. This reparative dentinogenesis mediated by newly generated odontoblasts that arise from pulp tissue is indicative of the presence of odontogenic progenitor or stem cells in the dental pulp.

Stem cells in dental pulp reside in a specific perivascular microenvironment or 'niche', where they are quiescent and maintain their basic stem cell characteristics, including a capacity for self-renewal and multipotentiality.[4]

In the present study, we have standardized the procedures for the isolation, culture and phenotypic characterization of stem cells from permanent and deciduous teeth - DPSC and SHED, respectively.

We prepared and used Mesenchymal Stem Cell Medium (MSC Medium) - α-modified minimal essential medium (α-MEM) after sterilizing the culture medium by filtration through a 0.22 µm membrane filter. 100 U/ml penicillin, and 100 µg/ml streptomycin was added to the medium. We supplemented the stock media with 15% Fetal Bovine Serum (FBS) for all cultures, as recommended by Freshney.[23]

Gronthos *et al*. (2000) [49] (2002) [47] (2003) [4], Huang *et al*. (2008)[27], Miura *et al* (2003) [48] cultured dental pulp stem cells in media supplemented with 20% FBS, while Suchanek *et al* (2010) [53] reported the use of 2% FBS in their cell culture studies. Serum contains growth factors which promote cell proliferation.[44] However, increasing the concentration of serum also makes the medium more susceptible to microbial contamination.

Takeda *et al* (2008)[50] lost nine of their DPSC cultures to bacterial/fungal contamination. The dental pulps of impacted permanent teeth are not exposed to oral flora while the pulps of deciduous teeth can be contaminated because of exposure of the resorbed areas to bacteria in the oral cavity. It is therefore critical, for successful cell isolation, to disinfect the tooth with a disinfectant reagent (we used D- PBS with double strength antibiotics) after extraction, just before isolation of pulp tissue. Despite the use of antibiotics and following aseptic conditions, three of our SHED cultures succumbed to bacterial contamination. Thus, one of the major issues that determine the use of deciduous teeth is the pulp being exposed to oral flora and the consequent susceptibility of the culture to microbial contamination.

The culture protocol that we employed was based on the ability of stem cells to adhere to culture dishes and form discrete colonies. Shi and Gronthos (2003) [4] hypothesized that stem cells are present in the perivascular niche of the dental pulp. We followed the technique put

forward by Freshney [23] and cut the pulp into small fragments using a sterile scalpel blade and disaggregated the bits using enzyme digestion to release the DPSC and SHED from their perivascular niche in the pulp tissue.

The duration of enzyme disaggregation has to be carefully controlled. If the enzyme digestion lasts for too long, it is harmful to the cells, so standardization of the duration of enzyme disaggregation to allow for the release of cells from the tissue and at the same time avoiding cell damage is important.[44] Initially, we digested the pulp tissue in enzyme solution (2mg collagenase and 1mg dispase solution in 1ml of Dulbecco's Phosphate Buffered Saline) for 60- 45 minutes. The duration had to be varied based on the amount of tissue obtained from the tooth. Deciduous teeth have considerably less amount of tissue as compared to the third molars and consequently need to be subject to enzyme digestion for a shorter span of time. Gronthos *et al*. (2000), [49] (2002),[47] (2003),[4] Huang *et al*. (2008),[27] used 3mg/ml of collagenase and 4mg/ml of dispase, for sixty minutes, for the digestion of cells from permanent tooth pulp. Suchanek *et al*[53] carried out enzyme disaggregation with collagenase and dispase, for 35minutes, for the pulp of both permanent and deciduous teeth. Miura *et al* (2003)[48] used 3mg/ml of collagenase and 4mg/ml of dispase in D PBS, for sixty minutes, for the digestion of cells from deciduous tooth pulp. Freshney described the digestion of pulp tissue using 2mg of collagenase and 1mg dispase in DPBS for 30-60minutes.[23] These varying values reported by investigators Gronthos *et al*. (2000)[49]

(2002) [47] (2003) [4], Huang *et al*. (2008) [27], Suchanek *et al* [53] (2010), Miura *et al* (2003) [48] and Freshney, [23] can be attributed to the modifications based on the difference in the amount of pulp obtained from each tooth.

During the course of the study, we found that the cells obtained were fewer when the duration of enzyme digestion was increased, but paradoxically, the yield of cells was again low when we decreased the time duration. To address this problem, we used a single enzyme-collegenase (3mg) and dissolved it in the culture medium (α-MEM), instead of in D PBS as the culture medium retards enzyme action and has a protective effect on the cells, preventing their digestion. To allow for the enzyme action to take place, we increased the duration of incubation of the cells in enzyme solution to 16-18 hours. This digestion of pulp tissue in enzyme dissolved in the culture medium instead of in D PBS is a modification in the methodology that we incorporated inorder to optimize the yield of cells from the tissue.

The pH of the culture medium is critical to the growth of stem cells. Both, the amount of carbon dioxide in the incubator, where the cells are incubated and the constitution of the culture medium, have a bearing on the pH. [44]

Carbon dioxide in the gas phase dissolves in the culture medium, establishes equilibrium with HCO_3^- ions in the medium, and lowers the pH. An increase in the amount of carbon dioxide reduces the pH, so high carbon dioxide tension has to be compensated by increasing the

bicarbonate (HCO_3^-) concentration, to maintain pH at appropriate levels (7.4-7.7). [44] To achieve this, we had to raise the pH of the stock media to 8.4, so that the pH was maintained at around 7.6 after the addition of 15% FBS and the action of carbon dioxide in the incubator (~5%) on the medium. This was achieved by the addition of 1N Sodium Hydroxide. The percentage of carbon dioxide used was similar to that reported by Gronthos *et al* (2000) [49] (2002) [47] (2003) [4], Miura *et al* (2003) [48], G T J Huang *et al* (2006) [46], Lopez- Cazaux *et al* (2006) [45], Takeda *et al* (2008) [50], Huang *et al* (2008) [27] and Shuchnek *et al* (2010)[53] for dental pulp cells.

Culturing the cells at a pH of 7.4-7.7 helped obtain a better yield of cells in the primary culture from permanent teeth. Deciduous tooth pulp was of very low quantity to begin with (cultures 1, 2 and 3 of deciduous teeth) and did not yield cells in primary culture after enzyme digestion. Shuchanek *et al* (2010) [53] also reported a low yield of cells in SHED cultures.

The explant/outgrowth technique was attempted with deciduous tooth pulp to avoid the risk of losing cells to enzyme action. The pulp tissue was dissected with a scalpel, covered with media containing 40% FBS and antibiotics and incubated overnight, as described by Freshney.[44] After twenty-four hours, we observed that the medium was covered with a white film of fungal spore and hyphal forms. The high concentration of serum and lack of enzyme action had made the environment fertile for microbial growth. G T JHuang *et al* (2006) [46]

used the explant method to culture dental pulp cells. They placed the pulp tissue explants (2×2×1 mm fragments) in 6-well plates with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with FBS and antibiotics. They did not report any fungal contamination of the explant culture. We did not continue using the explant technique because of the risk of fungal contamination.

To overcome the problem of deciduous tooth pulp having less amount of tissue, we decided to use two simultaneously extracted teeth (canines) from the same subject for a single primary culture. On the third day following plating of the cells, more number of cells were seen, equivalent to that seen in permanent tooth pulp cultures. This alteration in the methodology helped us obtain a better yield of cells from deciduous tooth pulp.

We analyzed the phenotypes of the cultured cells from permanent tooth pulp using the model proposed by Mollenhauer and Bayreuther (1986).[24] The description of the spindle shaped, fibroblastoid cell sub-populations as F1, F2, and F3 was based on the fibroblast-sub-population description done by Mollenhauer and Bayreuther (1986) [24] on the rat skin model and Bayreuther *et al* (1988) [25] for human skin fibroblasts as it was being followed in our laboratory for previous fibroblast culture studies. F1 is a small spindle shaped cell, F2 is a small epithelioid and F3 is a large pleomorphic and epithelioid cell. The ratio between F1, F2 and F3 sub-populations was determined. In the 14th permanent tooth pulp cell line there was a statistically

significant increase in the F3 cell type during the eight day observation period. The decrease in the F1 sub-population was not statistically significant. The 15th permanent tooth pulp cell line, showed an increase in the F2 and F3 sub-populations and a statistically significant decrease in the F1 sub-population over the eight day observation period. As this model was originally proposed for the study of rat skin fibroblasts and the fact that dental pulp stem cells do not follow distinct steps of sequential differentiation as seen in rat fibroblasts, the use of this model to study the cell phenotypes in mesenchymal stem cells from the dental pulp did not seem relevant. The graphs plotted to study the sub-populations also showed a daily increase followed by a decrease in the number of F1, F2, and F3 cells. In the light of this preliminary finding, we feel that this model may not be appropriate to study stem cells from dental pulp.

To study the difference in the morphology of DPSC and SHED, we classified ten cells (randomly chosen), as fibroblastoid if they had a thin, spindle-shaped morphology or "epithelioid"[23, 24, 25] if they were round and resembled epithelial cells. This was done for a period of fifteen consecutive days, starting from the sixth day after primary culture. We found that the cells from the permanent tooth pulp showed a higher proportion of spindle-shaped fibroblastoid cells whereas a higher proportion of epithelioid cells were seen in the deciduous pulp culture. This difference was statistically significant. Epithelioid cells are considered as contaminating cells during the isolation and propagation of mesenchymal stem cells.[23] The higher proportion of

epithelioid cells in deciduous tooth pulp cultures could be due to the open apex of the deciduous tooth used for culture. These findings also show that the DPSC and SHED populations are morphologically distinct. Our findings are consistent with Suchanek *et al* (2010)[53] who reported that SHED generated more rounded cells 'without long processes' as compared to DPSC. This difference in fibroblastoid:epithelioid cell ratio between DPSC and SHED may reflect the difference in osteogenesis/odontogenesis between DPSC and SHED as observed by Miura *et al*.[48] Deciduous teeth are significantly different from permanent teeth with regards to their developmental processes, tissue structure, and function.[48] Therefore, it is not surprising to find that SHED and DPSC show distinctly different ratios of fibroblastoid: epithelioid cells with respect to their cell morphology.

We studied the growth characteristics of the DPSCs and analyzed the derivatives obtained from the growth curve. A growth curve for a cell line shows a lag phase, which is the time taken for the cell to adapt to the new environment. Log phase is the time when cells start to divide in an exponential manner. The growth curve then reaches a plateau phase when the cells reach confluency.[44] The slope of the growth curve in the log phase yields the population doubling time (PDT). PDT derived from the growth curve is not equivalent to cell cycle time or cell generation time. PDT is an average value from the whole population of cells which includes dividing cells, non-dividing cells and dying cells, so PDT can be influenced by non-dividing and dying cells as well. [44] In our study, we found the population doubling

time to be 26 hours and 27 hours for DPSC cultured from the 14th and 15th permanent tooth pulps, respectively. Our findings are similar to that reported by Suchanek *et al* (2010)[53], in their study of DPSC, with a population doubling time of 24.5 hours. Suchanek *et al* (2010)[53] reported a doubling time of 41.3 hours for SHED. However, Miura *et al*[48] found that SHED had a shorter population doubling time as compared to DPSC indicating that they may represent a population of stem cells that are perhaps more immature than DPSC. [48] The population doubling time of the SHED (10th deciduous tooth pulp) cell line that we derived from its growth curve was 22 hours. As this value of was obtained from a single cell line (10th deciduous tooth pulp), it needs to be corroborated with the findings from more cell lines before a comment can be made on the doubling time of SHED vs. DPSC.

Seeding efficiency determines the percentage of cells getting attached to the plate after 12 hours of plating the cells. [44] We determined the seeding efficiency of the cell lines- 88.9% (14th permanent tooth pulp), 91.7% (15th permanent tooth pulp) and 84.25% (10th deciduous tooth pulp).

During primary culture of the dental pulp, we found that the cells proliferated as cell clusters/colonies. The cells within each colony were characterized by a typical spindle shape or fibroblast-like morphology. Gronthos *et al* (2000) [49] (2002) [47] (2003) [4], Miura *et al* (2003) [48], G T J Huang *et al* (2006) [46], Lopez- Cazaux *et al* (2006) [45], Takeda *et al* (2008) [50], Huang *et al* (2008) [27] and Shuchnek *et al*

(2010)[53] also reported the proliferation of stem cells as colonies during primary culture. Huang *et al* (2008) [27] described a procedure to study the colony forming efficiency of the cultured stem cells. They performed a clonal assay of the third passage cells and reported a colony forming efficiency of 72% for DPSC. Although clonal assays have not been reported by other investigators, we attempted to reproduce the procedure described by Huang *et al* (2008).[27] We carried out all our cell culture studies on cells obtained between the 3rd and 5th passages in order to ensure the availability of adequate number of cells for the analysis. We used fifth passage DPSC for the clonal assay. The average colony forming efficiency of the DPSC (14th deciduous tooth pulp) was 17.1%. The lower percentage obtained may be due to the loss of attachment of the initially seeded cells and the formation of colonies less than 2mm in diameter, that were not enumerated in the colony-count used to calculate the colony forming efficiency.

The data presented in our study shows that the post-natal dental pulp when cultured in Mesenchymal Stem Cell (MSC) medium, yields cells that are highly proliferative and have low population doubling time - 22 hours for SHED (10th deciduous tooth) and 26 hours and 27 hour for DPSC (14th and 15th permanent tooth pulp cell lines, respectively). The seeding efficiency of DPSC- 88.9% (14th permanent tooth pulp) and 91.7% (15th permanent tooth pulp) was higher as compared to SHED- 84.25% (10th deciduous tooth pulp).The cells derived from permanent and deciduous tooth show distinctly different ratios of fibroblastoid: epithelioid cells. The permanent tooth pulp

showed a higher proportion of spindle-shaped fibroblastoid cells while deciduous tooth pulp had a higher proportion of epithelioid cells.

In our study, although SHED had a population doubling time similar to that of DPSC, the seeding efficiency was lower than that of DPSC indicating that permanent tooth pulp may be easier to culture and a better source of stem cells.

Our study shows that DPSC and SHED are viable sources for stem cells. However, as there are phenotypic differences between DPSC and SHED, this needs to be studied further to understand its impact with respect to clinical application.

- Protocol for the culture of mesenchymal stem cells from the dental pulp of permanent and deciduous teeth using α-MEM (Mesenchymal Stem Cell media) was standardized.

- The population of cells derived from permanent teeth (DPSC) and dedciduous teeth (SHED) showed distinctly different ratios of fibroblastoid:epithelioid cells. The cells from the permanent tooth pulp showed a higher proportion of spindle shaped fibroblastoid cells whereas a higher proportion of epithelioid cells were seen in the deciduous pulp culture. The difference was statistically significant at 5% level, $p<0.05$.

- The estimation of growth curve and population doubling time was done for two DPSC cell lines (26 hours, 27 hours) and one SHED cell line (22 hours).

- The seeding efficiency of DPSC was 88.9% (14^{th} permanent tooth pulp) and 91.7% (15^{th} permanent tooth pulp) and was higher as compared to SHED which was 84.25% (10^{th}deciduous tooth pulp)

- Colony formation is a feature of stem cells. The colony forming efficiency of DPSC was 17%.

Permanent and deciduous teeth are viable sources of stem cells. The permanent teeth are easier to culture because of a lower chance of contamination with oral microflora. The growth characteristics of the cells obtained from both these sources are similar. However, there was a difference in the proportion of fibroblastoid: epithelioid cells between the cultures obtained from the permanent and deciduous teeth. This needs to be studied using specific markers to ascertain if they are indicative of two distinct populations or just morphologic variations representative of different stages of growth.

1. **Reya T, Morrison SJ, Clarke MF and Weissman IL.**

   Stem cells, cancer, and cancer stem cells

   Nature, 2001; 414:105-111

2. **Lovell-Badge R.**

   The future for stem cell research

   Nature, 2001; 414:88-91

3. **Hipp J, Atala A.**

   Sources of stem cells for Regenerative Medicine

   Stem Cell Rev, 2008; 4:3–11

4. **Shi S and Gronthos S.**

   Perivascular Niche of Postnatal Mesenchymal Stem Cells in Human Bone Marrow and Dental Pulp.

   Journal of Bone and Mineral Research, 2003; 18:696-704

5. **Bianco P and Robey PG.**

   Stem cells in tissue engineering.

   Nature, 2001; 414:118-121

6. **Doherty M J, Ashton B A., Walsh S, Beresford J N.**

   Vascular Pericytes Express Osteogenic Potential *In Vitro* and *In Vivo*

   Journal of Bone and Mineral Research, 1998; 13(5):828-838

7. **Fuchs E, Tumbar T**

   Socializing with Neighbours: Stem cells and their Niche

   Cell, 2004; 116; 769-778

8. **Moore K A., Lemischka I R**

   Stem Cells and their Niches

   Science,2006; 311; 1880-1885

9. **Gronthos S, Graves S.E, Ohta S, Simmons PJ**

   The STRO-l+ Fraction of Adult Human Bone Marrow Contains the Osteogenic Precursors

   Blood, 1994; 84, No 12; 4164-4173

10. **Gronthos S, Simmons P.J.**

    The biology and application of human bone marrow stromal cell precursors

    J. Hematother, 1996; 5:15–23

11. **Owen, M., Friedenstein, A.J.**

    Stromal stem cells: marrow-derived osteogenic precursors

    *Ciba Found. Symp.*1988, 136:42–60

12. **Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R.**

    Multi-lineage potential of adult human mesenchymal stem cells

    Science, 1999, 284:143–147

13. **Prockop D.J.**

    Marrow stromal cells as stem cells for non-hematopoietic tissues

    Science, 1997, 276:71–74

14. **Simmons P.J., Torok-Storb B**

    Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1

    Blood, 1991, 78:55–62

15. **Sonoyama, W., Coppe, C., Gronthos, S., Shi, S.**

Skeletal stem cells in regenerative medicine

Curr. Top. Dev. Biol., 2005, 67:305–323

16. **Friedenstein, A.J.**

Precursor cells of mechanocytes

Int. Rev. Cytol., 1976, 47:327–359

17. **Friedenstein, A.J., Chailakhjan, R.K., Lalykina, K.S**

The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells

Cell Tissue Kinet., 1970, 3:393–403

18. **Nagaya N, Fujii T, Iwase T**

Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis

Am J Physiol Heart Circ Physiol, 2004, 287:2670-2676

19. **Nosrat I V, Widenfalk J, Olson L, Nosrat CA**

Dental Pulp Cells Produce Neurotrophic Factors, Interact with Trigeminal Neurons *in Vitro*, and Rescue Motor neurons after Spinal Cord Injury

Developmental Biology, 2001, 238**,** 120–132

20. **Mao J J ,Giannobile W V,Helms JA, Hollister S J , Krebsbach P H , Shi S**

Craniofacial Tissue Engineering by Stem Cells

J Dent. Res., 2006; 85; 966

21. **Alhadlaq A, Mao J J**

Tissue-engineered Neogenesis of Human-shaped Mandibular Condyle from Rat Mesenchymal Stem Cells

J Dent Res., 2003; 82; 951

22. **Seo B M, Miura M, Gronthos S, Bartold P M, Batouli S, Brahim J, Shi S**

Investigation of multipotent postnatal stem cells from human periodontal ligament

Lancet 2004; 364: 149–55

23. **Freshney I R, Stacy G N, Auerbach J M**

Culture of Human Stem Cellls

Wiley- Interscience, 2007, 193-203

24. **Mollenhauer J and Bayreuther K.**

Donor-age-related changes in the morphology, growth potential, and collagen biosynthesis in rat fibroblast subpopulations *in vitro*

Differentiation, 1986; 32:165

25. **Bayreuther K, Rodemann H P, Hommel R, Dittmann K, Francz P.I**.

Human skin fibroblast *in vitro* differentiates along a terminal cell lineage

Proc. Natl. Acad. Sci. USA, 1988, 85: 5112

26. Van Wyke C W, Oliver A, Helden C G H, Grobler-Rabie A F

Growth of oral and skin fibroblasts from patients with oral submucous fibrosis

J Oral Pathol.Med., 1995,24, 349-353

27. Huang A H, Chen Y, Lin L, Sheih T, Chan A W

Isolation and characterisation dental pulp stem cells from a supernumerary tooth

J Oral Pathol. Med., 2008, 37; 571-574

28. Hamburger V

Wilhelm Roux: visionary with a blind spot

J Hist Biol., 1997 Summer; 30(2):229-38

29. Nicholas J S

Ross Granville Harrison, 1870-1959

Yale J Biol Med. 1960 Jun; 32:407-12

30. Carrel A.

On the Permanent Life of Tissues Outside of the Organism

J Exp Med. 1912 May 1; 15(5):516-28

31. Eagle H

Nutrition needs of mammalian cells in tissue culture

Science, 1955 Sep 16; 122(3168):501-14

32. Wade N.

Hybridomas: the making of a revolution

*Scienc*e, 1982 Feb 26; 215(4536):1073-5

33. Skloot R

The Immortal life of Henrietta Lacks

Crown Publishers, 2010, First edition

34. Robey PG and Bianco P

The use of adult stem cells in rebuilding the human face

JADA, 2006; 137:961-972

35. Ivanovski S, Gronthos S, Shi S and Bartold P M

Stem cells in the periodontal ligament.

Oral diseases, 2006; 12:358-363

36. Tavassoli M, Crosby W H

Transplantation of Marrow to Extramedullary Sites

Science, 1968, 161; 54-56

37. Friedenstein A. J

Osteogenic stem cells in bone marrow

Journal of Bone and Mineral Research, 1990, 243–272

38. Huang G T J, Gronthos S, Shi S

Mesenchymal Stem Cells Derived from Dental Tissues vs. Those from Other Sources: Their Biology and Role in Regenerative Medicine

J Dent Res, 2009; 88; 792

39. Scadden DT

The stem-cell niche as an entity of action

Nature, 2006, 441:1075-1079

40. Jones DL, Wagers AJ

No place like home: anatomy and function of the stem cell niche

Nat Rev Mol Cell Biol, 2008, 9:11-21.

41. Chen SC, Marino V, Gronthos S, Bartold PM

Location of putative stem cells in human periodontal ligament

J Periodontal Res, 2006, *41:547*-553

42. Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I

Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling

J Cell Biol, 1991, 147: 105-120.

43. Mitsiadis TA, Barrandon O, Rochat A, Barrandon Y

Stem cell niches in mammals

Exp Cell Res, 2007, 313: 3377-3385.

44. Freshney I R

Culture of Animal Cells- A Manual of Basic Technique

Wiley –Liss Publication, fifth edition

45. Lopez-Cazaux S, Bluteau G, Magne D, Lieubeau B, Guicheux J, Licht

Culture Medium Modulates the Behaviour of Human Dental Pulp derived Cells: Technical Note

European Journal of Cells and Materials, 2006,(11), 35-42

46. Huang G T, Sonoyama W, Chen J, Park S.H

In vitro characterization of human dental pulp cells: various isolation methods and culturing environments

Cell Tissue Res., 2006, 324:225–236

47. Gronthos S, Brahim J, Fisher L W, Cherman N, Boyde A, Robey, Shi S

Stem cell properties of human dental pulp stem cells

J. Dent. Res., 2002, 81:531–535

48. Miura M, Gronthos S, Zhao M, Lu B, Fischer L W, Robey PG, Shi S

SHED: Stem cells from human exfoliated deciduous teeth

Proc. Natl. Acad. Sci. USA, 2003, 100:5807–5812

49. Gronthos S, Mankani M, Brahim J, Robey P.G, Shi S

Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*

Proc. Natl. Acad. Sci. USA, 2000, 97:13625–13630

50. Takeda T, Tezuka Y, Horiuchi M, Honosa K, Tezuka K

Characterization of Dental Pulp Stem Cells of Human Tooth Germs

J Dent Res 2008, 87(7):676-681

51. Xiao Y, Mareddy S, Crawford R

Clonal Characterization of Bone Marrow Derived Stem Cells and Their Application for Bone Regeneration

Int J Oral Sci, 2010, 2(3): 127–135

52. Lehmann J M, Riethmuller G, Johnson J P

MUC 18, a marker for tumor progression in human melanoma, shows sequence similarity to neural cell adhesion molecule of immunoglobulin super family

Proc. Natl. Acad. Sci. USA, 1989, 86(4):9891–9895

53. Suchanek J, Visek B, Soukup T, Omran A

Stem Cells from Human Exfoliated Deciduous teeth- Isolation, Long term Cultivation and Phenotypical Analysis

Acta Medica 2010; 53(2), 93-99

54. Hu B, Nadiri A, Bopp-Küchler S, Perrin-Schmitt F, Lesot H

Dental Epithelial Histomorphogenesis *in vitro*

J Dent Res., 2005 84(6), 521-525

55. Dualibi MT, Dualibi SE, Young CS, Barlett JD, Vacanti JP, Yelick PC.

Bioengineered Teeth from Cultured Rat Tooth Bud Cells

J Dent Res, 2004; 33(7):523-528

56. Robey PG and Bianco P

The use of adult stem cells in rebuilding the human face

JADA, 2006; 137,961-972

ANNEXURE I
INFORMED CONSENT FORM

TITLE OF RESEARCH

Culture and Growth Characterization of Human Mesenchymal Stem Cells from Dental Pulp

NAME AND DESIGNATION OF THE PRINCIPAL INVESTIGATOR-

Dr Revathi. S
Post graduate student
Department of Oral and Maxillofacial Pathology,
Ragas Dental College & Hospital.

ABOUT THE RESEARCH

This study is being done to isolate, culture and study the yield of mesenchymal cells from the dental pulp of exfoliated deciduous and permanent teeth. The teeth collected after the patient has undergone extraction, will be used to obtain the pulp tissue. This pulp tissue will be used for cell culture. The growth characterization of cells isolated from the dental pulp of the permanent third molars will be evaluated for population doubling time and the phenotype of the cells will be analyzed.

A total of 10 teeth will be used for this study, over a period of 10 months. The participant will be asked questions about his age, chief complaint and medical history. All of this information will be kept confidential. The patient's extracted tooth will be used for further research.

CONFIDENTIALITY

Records will be kept in a safe and secure place with access limited only to the Principal Investigator or any other research staff. Publications if any will not identify the study participants.

RESEARCH RELATED INJURY

If the research participant is injured as a result of being in this study, he/she will be given immediate treatment for the injuries.

RISKS AND BENEFITS

- There are no risks or hazards to the study participants.
- Patient will also be given oral hygiene instructions.
- Researcher will bring to the notice of the patient any abnormal findings, observed in the study period.
- No monetary incentives will be provided.

Participation in this study is completely voluntary. The patient will be treated in the same manner irrespective of his participation in this study. In case of any problem or doubts regarding the study, patient can contact Dr Revathi.S (9790811492).

SIGNATURE PAGE

If you have read the informed consent (or if you have had it explained to you) and understand the information, and you voluntarily agree to take part in this study, please sign your name below.

----------	----------	----------
Volunteer's Name (typed or printed)	Volunteer's Signature	Date

OR

----------	----------	----------
Volunteer's Legal Guardian or Representative	Legal Guardian's Signature	Date

----------	----------	----------
Witness's Name	Witness's Signature	Date

----------	----------	----------
Interviewer’s Name	Interviewer’s Signature	Date

jftywpe;j Xg;Gjy;

Muha;r;rpapd; jiyg;G

jw;fhypf kw;Wk; epiyahd kdpj gw;fspd; kpnrd;ifky; itu];-d; tsh;r;rp Fzhjpraq;fs;> jd;ik kw;Wk; mjd; epiyg;Gj; jd;ik gw;wp Muha;jy;.

Kjd;ik Ma;thsh;

lhf;lh; Nutjp. v];

Xuy; kw;Wk; khf;]pNyh/Ng\pay; ghj;jhy[p Jiw

uhfh]; gy; kUj;Jtf; fy;Y}hp kw;Wk; kUj;Jtkid

Muha;r;rp gw;wp

epiyahd kw;Wk; epiyaw;w gw;fspy; kpnrd;ifky; itu];-ir jdpikg;gLj;jp> mjd; Fzhjpraq;fis fz;lwptjw;fhf ,e;j Ma;T elj;jg;gLfpwJ. Nehahspfspd; ePf;fg;gl;;l gw;fis Nrfhpj;J> mg;gw;fspd; rijfis cgNahfg;gLj;jg;gLfpwJ. itu]; tsh;r;rpapd; jd;ikia mDkhdpg;gjw;F ,e;j Muha;r;rp cl;gLj;jg;gLfpwJ.

10 khj fhyj;jpy;> nkhj;jk; 10 gw;fs; ,e;j Ma;Tf;fhf Nrh;f;fg;gLfpwJ. gq;Nfw;ghsh;fsplk;> mth;fsJ taJ> thapYs;s gpur;ridfs; kw;Wk; kUj;Jt tuyhW gw;wpa Nfs;;tpfs; Nfl;fg;gLk;. ,t;tptuq;fs; midj;Jk; ,ufrpakhf ghJfhf;fg;gLk;. Nkw;nfhz;L Muha;r;rpf;fhf epf;fg;gl;l gw;fs; Nrfhpf;fg;gLk;.

,ufrpaj;jd;ik

Kjd;ik Ma;thsh; my;yJ NtW ahNuDk; Muha;r;rpg; gzpahsh;fs; kl;LNk mZff;$ba tifapy; gjpTfs; ,ufrpakhd kw;Wk; ghJfhg;ghd ,lj;jpy; itf;fg;gLk;. VNjDk; ntspaPLfs; ,Uf;Fkhdhy;> mtw;wpy; Ma;Tg; gq;Nfw;ghsh;fs; milahsk; fhl;lg;glkhl;lh;fs;.

Muha;r;rp njhlh;ghd fhak;

,e;j Ma;tpy; ,Ug;gjd; fhuzkhf Muha;r;rp gq;Nfw;ghsUf;F fhak; Vw;gl;lhy;> mf;fhaq;fSf;F mtUf;F cldbahf rpfpr;ir mspf;fg;gLk;.

,lh;fs; kw;Wk; ed;ikfs;

- Ma;Tg; gq;Nfw;ghsh;fSf;F ,lh;fNsh my;yJ jPq;Nfh Vw;gl tha;g;gpy;iy.
- tha; Rfhjhuk; Fwpj;j mwpTiufSk; Nehahspf;F mspf;fg;gLk;.
- MaTf; fhyj;jpy; fz;lwpag;gLk; VNjDk; mrhjhuz epiyfs; Fwpj;J Muha;r;rpahsh; Nehahspf;F jfty; njhptpg;ghh;.
- nuhf;f Cf;fj;njhiffs; vJTk; toq;fg;glkhl;lhJ.

,e;j Ma;tpy; gq;Nfw;ghsh;fs; Kw;wpYk; RatpUg;gjpdhyhdJ. Nehahsp ,e;j Ma;tpy; gq;Nfw;whYk;> gq;Nfw;fhtpl;lhYk;> mth; xNu khjphp elj;jg;gLthh;. Ma;T gw;wpa VNjDk; gpur;rid my;yJ re;Njfq;fs; ,Ue;jhy;> lhf;lh; Nutjp v]; mth;fis (9790811492) Nehahsp njhlh;G nfhs;syhk;.

ifnaOj;Jg; gf;fk;

jftywpe;j xg;Gjiy ePq;fs; gbj;J (my;yJ mJ cq;fSf;F tpsf;fg;gl;L)> mt;tptuq;fis ePq;fs; Ghpe;J nfhz;L> kw;Wk; ePq;fs; ,e;j fz;fhzpg;G rhHe;j Ma;tpy; gq;Nfw;f RatpUg;gj;Jld; xg;Gf;nfhs;Sk; gl;rj;jpy;> jaTnra;J cq;fs; ngaiuf; fPNo ifnahg;gkplTk;.

jd;dhHtyhpd; ngaH (ilg; nra;jJ my;yJ mr;rpy;) my;yJ	jd;dhHtyhpd; ifnahg;gk;	Njjp
jd;dhHtyhpd; rl;lG+Ht ghJfhtyH my;yJ gpujpepjpapd; ngaH	rl;lG+Ht ghJfhtyhpd; ifnahg;gk;	Njjp
rhl;rpapd; ngaH	rhl;rpapd; ifnahg;gk;	Njjp
Ngl;bahshpd; ngaH	Ngl;bahshpd; ifnahg;gk;	Njjp

Protocol Version 1.0
9th October, 2009

Tamil Translation
9th October, 2009